ROLE OF BIOMARKERS IN EARLY DETECTION OF HETEROTOPIC OSSIFICATION FOLLOWING SPINAL CORD INJURY

Dissertation submitted to the Tamil Nadu Dr M.G.R Medical University, Chennai, Tamil Nadu, in partial fulfilment of the requirements for the MD branch XIX (Physical Medicine and Rehabilitation) University Examinations in April 2015.

Certificate

This is to certify that the thesis tilted **"Role of biomarkers in early detection of heterotopic ossification following spinal cord injury"** is the bone fide work of **Dr Vijay Kumar Manda**, candidate number **201229053** in partial fulfilment of the requirement of the Tamil Nadu Dr M.G.R Medical University, Chennai, Tamil Nadu for the MD branch XIX (Physical Medicine and Rehabilitation) University Examinations in April, 2015.

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By 201229053.md Physical Medicine A VIJAY KUMAR MANDA

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Place:

Date: **Dr. Vijay Kumar Manda**

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TITLE OF THE STUDY

Role of Biomarkers in Early Detection of Heterotopic Ossification following Spinal Cord Injury

Place of study

Department of Physical Medicine and Rehabilitation

Christian Medical College

Vellore.

AIM OF THE STUDY

Early detection of heterotopic ossification in spinal cord injury patients through biomarkers.

OBJECTIVES OF THE STUDY

1. To evaluate the sensitivity and specificity of CPK in early detection of heterotopic ossification in spinal cord injury patients.

2. To evaluate the sensitivity and specificity of osteocalcin in early detection of heterotopic ossification in spinal cord injury patients.

3. To compare serum CPK, osteocalcin levels in early detection of heterotopic ossification following spinal cord injury.

INTRODUCTION

INTRODUCTION

Spinal cord injury (SCI) is a partial or total disruption of the structural or functional integrity of the spinal cord following non-traumatic or traumatic cause leads to temporary or permanent impairment of motor, sensory and/or autonomic functions.

The spinal cord functions mainly in the transmission of neural signals between the brain and the target organs in the body but also contains neural circuits that can independently control numerous reflexes and central pattern generators. The disruption of these neurohumoral functions following spinal cord injury results in various acute and chronic secondary complications. There is a wide range of short and long term complications like Heterotopic Ossifications, spasticity, deep venous thrombosis, osteoporosis etc.

Heterotopic ossification (HO) is defined as 'formation of mature, lamellar bone in non-osseous tissue, usually between the muscle and the joint capsule'. This happens as a consequence of various conditions and trauma. These include neurological injuries like spinal cord injury, traumatic brain injury, post surgical intervention at the particular joint and direct injury to the muscle.

The exact cause of HO is not clear till date though the research has been well advanced in this field. The widely accepted concept is transformation of primitive mesenchymal cells which are present in the connective tissue into bone forming cells called osteoblasts when exposed to certain inducing agents(1). The exact sequence of events that lead to ectopic bone formation, the proper etiology and predisposing factors are still under exploration.

Early identification of this particular condition has always been a challenge. As the condition develops over a period of time with non specific complaints at the local site and occasionally mild constitutional symptoms; clinical suspicion needs to be tailored with proper investigation to achieve diagnosis at the earliest. It is well established that the medical treatment is most effective only when the patient receives it before the radiological evidence of bone formation. There are various laboratory biomarkers like alkaline phosphatase(ALP), creatine phosphokinase, osteocalcin and radiological tools including the use of ultra sound examination in early detection of HO have been reported in the literature. Three phase bone scan is widely accepted as Gold standard(2–4).

Currently pharmacotherapy is the mainstay of treatment for HO. Disodium Etidronate (Didronel), a non nitrogenous bisphosphonate was the drug of choice for both prophylaxis and treatment as it can slow down the mineralization process(5). Didronel is expected to be effective in early stage of the disease(3). Other medications like NSAIDs, Warfarin(6,7), glucocorticoids are also in use. Indomethacin is used most commonly which is shown to be effective in the prevention of HO especially when the patient receives during the first two months after the spinal cord injury even before the clinical features appear(8–10). Low field irradiation showed some response when used as the primary treatment once first clinical signs were observed(11–13). The surgical intervention is the last resort available when the HO becomes fully mature. The complication rate is high for the surgical resection which includes increased bleeding and recurrence.

JUSTIFICATION OF THE STUDY

JUSTIFICATION OF THE STUDY

HO is one of the complications following spinal cord injury. The precise mechanism of HO formation is not known. HO forms in the muscle planes primarily rather than muscle itself. Though the muscle fibers are not involved in the process of HO formation, they are incorporated in to the newly formed bone tissue or compressed by the adjacent fibrosing soft tissue calcification that leads to necrosis of the local muscle(14).

In spinal cord-injured patients the incidence of HO is between 20% and 25%(15). Increased joint stiffness, a limited range of motion, warmth, swelling and erythema are the main clinical signs of HO. The gold standard for the diagnosis of heterotopic ossification is three-phase bone scintigraphy. It is the most sensitive imaging technique for the early detection of HO. In the untreated patient the three-phase bone scan detects the formation of HO generally 4 to 6 weeks before ossification is noticed on the X-ray study. The serial three-phase bone scan can also be used as a reliable method of determining the maturity of HO(4).

Ultrasonography was also proved to be detecting the condition of HO sooner than the conventional radiography(16–18). Local ultrasonographic signs of inflammation like “zone phenomenon” and cystic changes in spinal cord injured patients are suggestive of heterotopic ossification. *Snoecx et al* demonstrated possible relationship between microtraumatic lesion and occurrence of HO by ultrasonographic changes(17). In a study done on patients with HO after total hip replacement, it was found that ultrasonography is also the probable investigative choice not only for the early identification of the condition, but also for the follow-up of HO(16). But the expertise makes the usage of sonography more difficult.

The primary investigations include the assessment of ALP levels and bone scintigraphy. The sensitivity of serum alkaline phosphatase which is elevating as an indicator of heterotopic bone has been a matter of disagreement in the literature (16–18). Scintigraphic demonstration of heterotopic bone can happen when the serum ALP is within normal limits though the levels of the same are rising pace below the upper limit(19). After Four weeks of injury alkaline phosphatase levels may reach up to 3.5 times the normal value, with a peak concentration around the 12th week. If HO formation is small, ALP levels may remain unchanged. This is a good parameter in the absence of fractures. An increase may indicate the functional transformation of mesenchymal stem cells into chondrocytes(15). *Kluger et al* showed in children there is no ALP increase during the development of heterotopic ossification(20).

Serum creatine phosphokinase (CPK/CK) is a marker of muscle injury. There are 3 iso-enzymes, described in the literature with the predominance of existence in muscle as CK-MM, CK-BB in brain and CK MB in heart muscle. The normal serum contains CPK MM activity 95-100% of the total CK. Hence it is legitimate to measure the CPK activity. The peak period for the rise in CPK level is 24hrs post injury(21). *Singh RS and Sherman et al* showed an elevation of serum CPK may be a more reliable subsequent HO predictor(22,23). Both these studies showed CPK as a potential predictive test for HO but they lack good study qualities such as inadequate sample size and low correlation with the people who developed HO. Hence CPK is included in the current proposed study.

Serum osteocalcin is released from the osteoblasts during the bone formation stage. In 1993, *Mysiw WJ* et al showed this biomarker may not contribute for the diagnosis of neurogenic heterotopic ossification once the clinical diagnosis has been established(24). But this study has done only on 12 traumatic brain injury patients and as there is significant improvement in the standard operating procedures of serum osteocalcin assay over past two decades, it is also included in the current study for the spinal cord injury population group.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Heterotopic ossification (HO) is one of the non life threatening complications following spinal cord injury which causes functional limitation. HO is described by *Dejerine and Ceillier* in 1918 as paraosteo arthropathy. It is defined as the formation of lamellar bone in periarticular non ossified soft tissue but without periosteal involvement(15,25). This bone has collagen which is arranged in regular parallel alignment of sheets which becomes mechanically strong eventually. Based upon the origin of ectopic bone, location, clinical presentation, isolated or associated with other conditions and progressive occurrence the condition of HO has been classified into 4 types, they are

(a) Post traumatic (following bone fracture, direct muscle trauma and surgical trauma).
(b) Neurogenic spinal cord injury, traumatic brain injury and very rarely in non traumatic CNS injury like stroke and Guillian- Barré syndrome.
(c) Myositis.
(d) Progressive fibro dysplasia ossificans.

Incidence

The range of the incidences of spinal cord injury in Asia was between 12.06 and 61.6 per million population. The incidence of traumatic spinal cord injury was lower than that in North America due to socioeconomic development. In all the countries the highest incidences were reported in people who were between 20 and 50 years. In Asia alone, majority of the adults with the range between 26.8 and 56.6 years are afflicted with the sequelae of spinal cord injury, due to their capability of being most dynamic and productive members of the society(26).

Incidence of HO, depending on the study and various institutions ranges from 20 to 30%(15,27). This discrepancy is also due to several other factors such as population studied, duration of the studies, the methods used for data collection and procedures adopted in different centers. Clinically significant heterotopic ossification usually develops in about 20% of patients with spinal cord injury. Out of these patients with HO, 8 to 10 percent develop severe functional limitations. Joint ankylosis was noticed only in 3 to 5 % of all these patients with HO in spinal cord injury(28,29). A correlation with age is suggested with heterotopic ossification being more common in patients between 20 to 30 years old. Although this may be related to the range of age at which spinal cord injury are most frequent. The incidence of HO in children with spinal cord injury was 3-10% and it was noticed that the spontaneous regression of HO happens more commonly in children and young adults(20,30).

Etiology and pattern of formation of HO

HO always occurs below the neurological level in the spinal cord injury(28,31). The increased spasticity may be seen associated with HO formation. But the causation of HO due to spasticity is still unknown(32). It has been strongly correlated with the degree of completeness of spinal cord injury and the direct trauma to the muscle(33). The association of HO formation and the spinal cord injury at cervical and thoracic level is more established than the occurrence of the same in the lumbar cord injuries(34,35). 97% of the HO happens in hip joint following spinal cord injury(29). In the hip joint the ossification of the tissues happens most commonly in the medial side extending from the symphysis pubis to the anteromedial aspect of the femoral shaft. Occasionally HO can even form posterior to the femoral neurovascular structures. In mature HO of hip joint it is also seen in anterior aspect involving iliopsoas with advancing lateral and

involving gluteus minimus posteriorly extending from ileum to encase the sciatic nerve(2). In knee joint HO, though very rare it happens anteriorly beneath the quadriceps muscle medially in the medial collateral ligament, posteriorly about the insertion of hamstring muscles.

Other joints that are involved in HO formation in the decreasing incidence are knee, elbow, shoulder, hand and spine.

A recent case control study of 264 patients by *Citak et al* showed there is a higher risk of developing HO in spinal cord injury patients with spasticity, thoracic trauma, pneumonia, urinary tract infection and presence of tracheostomy along with completeness of injury(36).

The influence of passive range of exercises on the risk of heterotopic ossification is so far unclear. But the role of micro trauma, mechanical stress to the muscles and the tendons appears to influence the bone formation. These may arise either from vigorous passive exercises or immobilization and the muscle imbalance causing the increased pressure on the soft tissue. This mechanical stress causes local micro trauma that may induce the bone formation. Michelson J. E. et al in a rabbit study showed increased duration of immobilization of the animal correlates the grade of HO where as five weeks of vigorous mobilization resulted in little or no HO formation(37).

In a recently done study by Christina et al to see the role of increased paratharmone levels in the formation of HO, a 12 fold increased ALP activity when BMP levels increased due to trauma or immobilization, proved hyperparathyroidism increases the risk of HO formation(38).

Race of the patient does not appear to be a significant factor for heterotopic ossification that occurs following spinal cord injury. A study done in 1992 about the sex predilection showed male spinal cord injury patients are twice as likely to develop HO as are female spinal cord injury patients(39). But a recent study showed there is no known sex predilection for neurogenic heterotopic ossification(5).

Pathophysiology

The formation of ectopic bone is similar to callus formation but it happens in the connective tissue between muscle planes. It is usually in the contiguity of the skeleton without periosteal involvement. Although the precise mechanism for the heterotopic ossification is largely unknown, there are few humoral, neural and local factors probably play a role in the pathophysiology(2). As the HO begins to form, the blood flow to the particular affected soft tissue will be increased along with the inflammatory reaction(40). The condition advances with cellular infiltration, fibroblastic proliferation, osteoid formation and deposition of bony matrix in a sequence. Ackerman noticed zone phenomenon during the maturation of new bone that occurs from outside to inside of the three zones. These three zones are thin outer zone of previously formed bone with well demarcated outer trabecular rim, intermediate zone with immature bone lined by osteoblasts and inner zone with undifferentiated fibroblasts studded in necrosed muscle tissue(41). Progressive mineralization happens at the junction between the outer and the intermediate zones which will eventually become radiologically opaque. The mature HO comprises of cancellous bone with Haversian canals, blood vessels, bone marrow and cortex. There are few histopathological studies that showed a minor amount of hematopoesis happening in this mature bone(28,40).

Chalmers(42) has proposed the existence of three conditions which are required for the HO formation. They are

(a) Presence of bone forming precursor cells which are primitive mesenchymal cells.

(b) A stimulus or an inducing agent that has been recently identified as bone morphogenic protein (BMP).

(c) The environment that permits osteogenesis.

Urist explored the role of BMP in 1997. He observed that the bone matrix which is demineralised can initiate new bone formation when supplied with BMP in non ossified tissue(43). The BMP is released from healthy bone tissue in the settings of inflammation, immobilization, trauma and venous stasis(38). There are various types of BMPs that are essential for the differentiation of mesenchymal cells into osteogenic cells of which BMP4 is important(15,44). Usually in normal conditions this BMP is under the action of antagonists such as gremlin, noggin, chordin and follistatin. The over expression of BMP4 happens with the decreased inhibitory response of these substances thus causing increase in the number of osteogenic cells(15).

There are mainly two factors that are responsible for ectopic ossification at cellular and molecular level (a) ischemic degeneration of involved muscles and (b) presence of BMPs. The sources of these BMPs in the muscle are mesenchymal cells and endothelial cells. This BMPs act upon the target cells which are mesenchymal cells and the smooth muscle cells. There is tenfold increase in the ALP activity when these multipotent mesenchymal stromal cells were stimulated

by BMP which can eventually become preosteoblastic cells(45). Canalis noticed there is only five fold ALP activity when BMP stimulated the mature osteoblastic cell. In his study he also observed BMP2 generally exhibits more expression than that of BMP3 which causes more increased ALP activity. In fact BMP 3 causes inhibition of osteogenesis(47).

In the early stage of HO formation, there are reported venous stasis, arterio-venous shunting and ischemic damage that cause the release of eicosanoids which are important factors in bone metabolism(15). There are many such inducing agents like insulin like growth factor II, tissue growth factor β, platelet-derived growth factor interleukin-1 and interleukin-6 which were observed in rat experiments. And these were shown to increase the osteoblastic activity in the animals with spinal cord injury. A 24 hour urine sample for PGE2 excretion and hydroxyprolein in spinal cord injured patients have a valuable indication for the early HO(48).

Medici and Olsen brought up a new concept of endothelial mesenchymal transition (EndMT) in which there is a dedifferentiation of cells from the vascular endothelium to a stem cell phenotype that eventually forms the bone, fat and cartilage. They proposed that these vascular endothelial cells are the primary factors for the cellular origin of HO. The vascular endothelial growth factor and several other EndMT- inducing transcription factors may serve as possible agents to arrest the HO formation and could be involved in the further therapeutic interventional studies(38,49).

Dejerine suggested the intermedio-lateral sympathetic columns of the damaged spinal cord cause the neurogenic heterotopic ossification through the autonomic dysregulation(28,50). Hohmann in 1986 in his study, found that bones along with periosteum are innervated by sympathetic vasoactive intestinal peptide (VIP) containing nerve fibers(51). Though the relationship between the bone formation and the nervous system is unclear it is well established that specific

neurotransmitters have a direct effect on bone metabolism. Other neurotransmitters like glutamate, substance *P*, calcitonin gene related protein and catecholamines also showed to increase the osteoblastic activity and at the same time reducing the osteoclastic activity(2). In another study, patients with brain injury and polytrauma produced more bony callus in the fracture site as part of healing than their counter part patients who did not have traumatic brain injury. The increased levels of catecholamine and sympathetic activity and the increased calcitonin may be the cause for more rapid healing of fractures(52–55).

Recent rat spinal cord injury models showed leptin, a hormone which plays important role in the regulation of energy homeostasis influences the bone formation by possible process of hypothalamus and sympathetic nervous system(56,57).

Histology

It is difficult to differentiate the heterotopic ossification from callus formation of a healing fracture histologically. The initiation of the ossification process is fibroblastic proliferation. The studies of histological aspects demonstrated a zone of fibroblastic metaplasia followed by chondroblast which subsequently transformed into osteoblast cells with blood vessels and Haversian system.

In the ectopic bone, mature lamellar bone is seen peripherally surrounded by compressed muscle fibers and connective tissue that form a capsule. Around the HO edema, muscle necrosis and osteoporosis with signs of hypersensitivity are also noted. These are considered to be consequences rather than cause of HO. Histological studies showed bone forms in connective tissue between muscle planes and not in the actual muscle itself(15). The histological picture of

the mature HO may depict cancellous bone, mature lamellar bone with blood vessels and bone marrow with demonstration of minute levels of haematopoiesis. In a study done by *Lotta et al* to describe the ultra structural and histological features of soft tissue and skin near the HO that formed in two spinal cord injury patients, showed there are changes in the endothelial cells and basement membrane of capillaries and small vessels. The endothelial cells showed hyperactivity whereas basement membrane showed thickness and reduplication. They concluded these vascular alterations may induce hypoxemic changes in the tissues around the joint that lead to metabolic changes which can lead to the development of HO(58).

There are few main histological stages in the usual evolution of HO following trauma which are described as follows

(a) Spindle cell formation that happens within the first week of trauma,

(b) Primitive osteoid stage that develops in 7 to 14 days

(c) Primitive cartilage and woven bone stage that can be seen in the second week,

(d) Trabecular bone formation at 2 to 5 weeks,

(e) Zone phenomenon which is immature, undifferentiated central tissues with mature peripherally located lamellar bone that happens approximately at 6 weeks.

(f) Mineralization in which hydroxyapatite crystals is formed by replacing the amorphous calcium phosphate. (59)

(g) True bone formation that happens over a period of approximately 6-18 months. This happens only in the muscle planes, not within the muscle without disrupting the joint capsule. It may attach to the adjacent bone cortex with or without damaging the same.

Clinical features

It is often difficult to diagnose heterotopic ossification in its initial stage. The symptoms and signs may appear as early as 3 weeks to 12 weeks after the injury. The earliest symptom of heterotopic ossification is usually stiffness and restriction of joint movements which progresses gradually. This is usually accompanied by swelling which eventually becomes indurated, with erythema, local rise of temperature, palpable mass and local pain (especially in the patients with incomplete spinal cord injury). It is not unusual to find contracture of the local muscles which can contribute for the further reduction of range of movements. Sometimes it is associated with mild to moderate grade fever. As the HO matures the local manifestations will slowly regress and eventually a hard, non tender bony lesion may arise approximately six to twelve weeks after the initiation of symptoms at the particular joint.

In children with spinal cord injury, decreased range of movement can be the only presentation without any other local symptoms and signs. In pediatric patients the average length of time reported between diagnosis of neurogenic heterotopic ossification and the spinal cord injury is around fourteen months whereas this duration in adult patients is six months(5).

The most common site for HO formation following spinal cord injury is hip joint(28,29) and the muscles which tend to be involved more frequently are hip flexors and abductors than hip extensors and adductors. The non articular manifestations are rarely noticed. Cases of nerve

entrapment around the joint and also vascular compression which is venous predominantly were noted. HO with associated increased spasticity can cause the obstruction of lymphatic vessels manifesting the features of lymphedema. Large amounts of heterotopic ossification can cause skin break down, pressure ulcers and inability to sit upright thus affecting the functional abilities especially transfers and difficulty wheel chair mobility.

The differential diagnosis of heterotopic ossification includes deep venous thrombosis (DVT), cellulites, septic arthritis, hematoma, osteomyelitis, tumor, fracture or local trauma. It is always difficult to differentiate clinically especially from lower extremity deep venous thrombosis because the same symptoms of swelling, erythema, pain can occur especially reason being immobilization as the causative factor for both conditions. Few studies showed DVT and heterotopic ossification have been associated positively, this might be due to the pressure effect and local inflammation of the ectopic bone promote the adjacent thrombus formation by causing compression of the nearby veins and causing phlebitis(60–64).

Natural course of HO

Most of the heterotopic ossification (around 80%) have relatively benign course leaving no complications. In the remaining 20% significant loss of motion develops with bony ankylosis. Among these, around 10% may cause severe disability eventually(3). It was noticed stabilized post traumatic HO, can spontaneously regress in few cases where as post SCI-HO usually does not regress without any surgical intervention(65). In 1999, a case of post burn heterotopic ossification transformation in to osteosarcoma was reported(66).

Diagnosis and classification of HO

Most of the patients can be suspected to have HO by the above mentioned clinical manifestations. In these patients, there is a necessity for the radiography to see the extent of the involvement of a specific joint. There are various methods of staging of heterotopic ossification mentioned in the literature. X-ray has been the important tool to grade HO.

Nicholas in 1973(67) proposed the staging of heterotopic ossification as shown in table 1

Table 1. Nicholas Staging of heterotopic ossification

Stage 1	Swelling, normal X- ray increased serum ALP
Stage 2	Swelling, positive X- ray, and increased serum ALP
Stage 3	No swelling, positive X- ray and increased serum ALP
Stage 4	No swelling, positive X- ray and normal serum ALP

In the same year 1973, Brooker et al (68) described heterotopic ossification classification around the hip joint following total hip replacement surgery on the basis of antero-posterior hip radiograph.

Table 2. Brooker et al grading of HO

Grade 0	No heterotopic ossification
Grade 1	Diffuse islands of bone within the soft tissues about the hip
Grade 2	Bone spurs with at least 1 cm gap in between the bony surfaces
Grade 3	Bone spurs with less than 1cm between the bony surfaces
Grade 4	Bony ankylosis of the hip joint

A recently proposed classification by *Mavrogenis* et al (69) in 2012 depicts the anatomical location of ectopic bone, bony ankylosis and etiology of the neurological injury(traumatic spinal cord injury or traumatic brain injury). This helps in the pre operative planning for the surgical approach for the excision of the same. To an extent this method may also be useful for the estimation of the intra operative complications like blood loss and transfusion requirements. Nonetheless this classification in the study done failed to show the significant relationship between the HO type and recurrence rate of the same.

Type with subtypes	**Description**
Type1 a) Spinal cord injury b) Traumatic brain injury	HO at the anterior aspect of the hip joint or proximal end of the femur; +/- ankylosis
Type 2 a) Spinal cord injury b) Traumatic brain injury	HO at the posterior aspect of the hip joint or proximal end of the femur; +/- ankylosis
Type 3 a) Spinal cord injury b) Traumatic brain injury	HO at the anterior and medial aspect of the hip joint or proximal end of the femur; +/- ankylosis
Type 4 a) Spinal cord injury b) Traumatic brain injury	HO at the circumferential aspect of the hip joint +/- ankylosis

Table 3. classification by *Mavrogenis* et al *(2012)*

IMAGING STUDIES

Radiography

The plain radiographs detect HO as early as around one to ten weeks after the first clinical signs in the patients with spinal cord injury, which is relatively a late finding. The increased density of the soft tissues around the joint is the earliest radiographic sign of HO. As the condition advances the calcium precipitates are seen as flocculent densities (tufts of wool). The formation of bony cortex with the trabeculations delineating from the soft tissue can be seen in the advanced stages of HO. In a long term follow up study, X-ray modality failed to show the maturity of HO(70). It could be possible for the immature bone is masked by the mature bone substance. This technique may be valuable if they show modification in sequential studies(40). It also failed to detect the possible reoccurrence or reactivation(71).

CT scan

CT imaging is a tool for the better visualization of HO among the soft tissues compared to plain X-ray. Typical findings of this investigation include an enlarged muscle belly, low attenuation of soft tissue mass(72,73). Single-photon emission computed tomography (SPECT) and three dimensional tomography (3D-CT) are proved to be of much use in planning the surgical resection of HO. This can avoid the immature bone being excised in order to prevent the recurrence(72).

MRI scan

Magnetic resonance imaging (MRI) is usually not recommended for the diagnosis of heterotopic ossification. The features of HO on MRI are low signal intensity rim and a tumor like enlargement of involved soft tissues with high signal intensity(74,75). A high cost and low specificity in diagnosis of early stage of HO make the MRI modality less preferable in regular practice(15).

Ultrasonography

Ultrasonography can detect HO earlier than the conventional radiography(16–18). "Zone phenomenon" and cystic changes in patients with spinal cord injury are suggestive of heterotopic ossification. *Snoecx* et al described possible relationship between microtrauma and occurrence of HO by USG changes(17). *Pistarini* et al in 1995 conducted a study on patients with HO after total hip replacement to see the sonography diagnostic value. They observed Ultrasonography was found to be the probable investigation of choice for both the early identification of the condition and for the follow-up of HO(16). But the expertise makes the usage of sonography more difficult. Usually it is not practiced for the routine patient care.

Triple phase bone scintigraphy

The three phase bone scan is considered to be the gold standard for early detection of HO as it has highest sensitivity for the localization of the same(2). It becomes positive typically during the third week following the spinal cord injury which allows detecting the condition four to six weeks earlier to plain X-rays. A remarkable finding of the ability of bone scan in differentiating

the callus formation from normal bone in experimental fractures in 1971 made a rapid advance in the technology and science of bone scan(76). This study also concluded the reliability of bone scan in monitoring the maturity of HO.

There are three phases during which the images are studied after the intravenous administration of radionuclide Technetium-99m.

Phase I (Blood flow phase): Detects areas of increased blood flow, an early sign of inflammation.

Phase II (Blood pool phase): Detects areas of increased static status; a phenomenon, several minutes after injection.

Phase III (Static bone phase): Determines the degree of osseous uptake of the labeled radionuclide several hours after injection.

First two phases are effective in detecting HO as early as 2.5 weeks after SCI followed by third phase around 1 week later. Rossier et al noticed the absence of vascularity as a sign of the maturity and may be planned for the surgical resection(40).

LABORATORY EVALUATION

Alkaline Phosphatase

The sensitivity of elevating serum alkaline phosphatase as an indicator of heterotopic bone has been a matter of disagreement in the literature (16–18). Scintigraphic demonstration of heterotopic bone can happen when the serum ALP is within normal limits though the levels of the same are rising pace below the upper limit(19). After Four weeks of injury alkaline phosphatase levels may reach up to 3.5 times the normal value, with a peak concentration around the 12th week. If HO formation is small, ALP levels may remain unchanged. This is a good parameter in the absence of fractures. An increase may indicate the functional transformation of mesenchymal stem cells into chondrocytes(15). Other major drawbacks of this investigation are normal levels do not correlate with the advanced stage of HO and peak level doesn't represent the peak activity of osteoblasts. A non specific elevation of ALP can happen if patient sustains skeletal injuries, liver injuries or receives surgical treatment for the fractures. *Kluger* et al showed in children there is no ALP increase during the development of heterotopic ossification(20).

Creatine Phosphokinase (CPK/CK)

Serum CPK is an intracellular enzyme. The disruption of cell membranes due to injury or hypoxia causes the release of CK from the cell into systemic circulation. There are 3 iso-enzymes, described in the literature with the predominance of existence in muscle as CK-MM , CK-BB in brain and CK MB in heart muscle.

CPK catalyzes the following reversible reaction by transferring phosphate group

ADP + Creatine Phosphate → ATP + Creatinine

The usual method of total CK measurement involves spectrometric determination of the rate of forward reaction. There are various modalities to separate CK into its isoenzymes like column chromatography, electrophoresis and radioimmunoassay of which electrophoresis is the most commonly used one in the clinical laboratories. The three isoenzymes behave as follows CK-MM – neutral, CK-MB – intermediate and CK-BB – most mobile

The normal serum contains CPK MM activity 95-100% of the total CK. The peak period for the rise in CPK level is 24hrs post injury(21). In the literature, CPK was well identified as a marker for muscle injury, induced muscle soreness due to vigorous exercises(21,77,78). Singh RS and Sherman et al showed an elevation of serum CPK may be a more reliable subsequent HO predictor(22,23). Both these studies showed CPK as a potential predictive test for HO but they lack good study qualities such as inadequate sample size and low correlation with the people who developed HO.

Osteocalcin

Serum osteocalcin is a bone-specific, vitamin K dependent, calcium binding, non-collagen protein in the bone matrix. The molecular weight of osteocalcin is approximately 5800 Dalton. It consists of 49 amino acids. Osteocalcin is also called as “bone Gla protein” because of the presence of three γ-carboxyglutamic acid residues. Osteocalcin is released from the osteoblasts during the bone formation stage. Its production is stimulated by vitamin D3. Once released, part

of the osteocalcin is incorporated in the bone matrix and remaining is delivered into the blood stream. In the blood, both carboxylated and under-carboxylated osteocalcin are present(79). In vitro experiments on primary adipocytes and isolated islets by *Lee NK et al*, it was noticed that the uncarboxylated form of osteocalcin is active and the carboxylated form is inactive(80). Osteocalcin is widely used as a bone turnover marker, as its level in serum can be related to the rate of bone turnover in many bone metabolism disorders (osteoporosis, primary hyperparathyroidism, secondary hyperparathyroidism, Paget's disease) especially in monitoring the therapeutic response. In blood, both intact osteocalcin (amino acids 1-49) and large N-Mid fragment (amino acids 1-43) are present. N-mid fragment resulting from cleavage is stable, whereas intact osteocalcin is unstable because of the protease cleavage between amino acids 43 and 44.

In 1993, Mysiw WJ et al showed this biomarker may not contribute for the diagnosis of neurogenic heterotopic ossification once the clinical diagnosis has been established. This study was conducted in traumatic brain injury patients(24).

MANAGEMENT

Pharmacological intervention:

NSAIDs:

Indomethacin (a non- selective COX inhibitor) is the drug used mainly in the prevention of heterotopic ossification. It should be initiated within two months of injury and continued for four to six weeks. Long acting indomethacin 75mg daily or standard release indomethacin 25mg thrice daily is suggested(9). In a study performed by *Banovac K et al* in 2004, when Rofecoxib was used as a primary prevention with a dosage of 25mg daily for four weeks, there was a significant decrease in the incidence of heterotopic ossification(81). But Rofecoxib was withdrawn from the market by FDA because of concerns about increased risk of developing myocardial infarction and cerebrovascular accidents based on VIGOR study analysis(2,81,82). The basic understanding of theses NSAIDs mechanism of action is inhibition of prostaglandins thus causing decreased mesenchymal cell differentiation into osteoblastic cells.

Bisphosphonates:

Disodium etidronate is a bisphosphonate which may inhibit the mineralization of organic osteoid when used intravenously(83). It is used in the primary prevention of heterotopic ossification in the early phase and to decrease the amount of bone formation in late phases. If etidronate is initiated after bone scan evidence, but before heterotopic ossification findings in plain films, HO can be prevented(84). Experiments carried out on dogs by *Peter CP et al* suggests that bisphosphonates delay callus remodeling which impairs fracture healing(85).

Radiation therapy:

Radiation prevents the differentiation of mesenchymal progenitor cells into heterotopic ossification forming osteoblasts(86). Radiation therapy given preoperatively is as effective as multi-fractionated post operative radiation therapy. In patients undergoing hip surgery radiation therapy prevents the formation of heterotopic ossification(87,88). However, a prospective randomized study by *Hamid N et al* showed increased risk of nonunion in elbow fractures when single- fraction therapy was administered(89). In a trial by *Sautter-Bihl ML* proved that there was no progression of heterotopic ossification but improvement in mobilization and rehabilitation when radiation therapy was administered following surgical resection of heterotopic ossification(90).

Physiotherapy:

Physiotherapy involves both active and passive ROM, gentle terminal stretch exercise and resisted ROM exercise. Though the aggressive exercises may increase the risk of HO formation, these exercises help in preventing movements and prevents ankylosis (91,92).

Surgical management:

Surgical resection of HO reduces pressure ulcers, impingement of important neurovascular structures and intractable pain and enhancing the function of mobility and giving care. Surgery is recommended one year following spinal cord injury(71). Alkaline phosphatase values, radiographs, 3D CT scan and bone scan data are to be considered before surgery. It is important to wait till maturity in order to minimize the recurrence rate following surgery. However

Freebourn TM et al suggest that resection of immature heterotopic ossification may not always have increased rate of recurrence(93). Delayed surgical intervention can lead to irreversible joint injury. The importance of timing for surgical intervention is to be investigated further. Recurrence is likely to be less in patients with good motor function and neurological recovery. In a case series by *Moore TJ*, patients who underwent surgical resection of heterotopic ossification and received concomitant bisphosphonate therapy, achieved 85° mean ROM for the hips and 65° mean ROM for the elbows(94). In another study, *Meiners T et al* observed that 94.5° mean ROM was for the hip joints was achieved, if physiotherapy and concomitant radiotherapy were given during immediate post operative period and was sustained to a mean of 82.7° after 4 years(95).

MATERIALS AND METHODS

MATERIALS AND METHODS

Study design

Prospective, diagnostic pilot study in a spinal cord injury patients' cohort group.

Setting for the study

The study was done in the Department of Physical Medicine and Rehabilitation (PM&R) of Christian Medical College Hospital (CMCH), Vellore. CMCH Vellore is a tertiary care centre located in Tamil Nadu, India. This centre has an inpatient capacity of about 2,700 beds. The number of patients attending OPD each day is approximately 5000. The PM&R department of CMCH, Vellore has approximately 130 beds and its outpatient department serves around 100 patients every day. Nearly 300 patients with spinal cord injury are rehabilitated in a year. Around 20-40% of these admissions are diagnosed to have heterotopic ossification.

This study was approved by the Institutional Review Board and Ethics committee of Christian Medical College and Hospital, Vellore.

Participants

Individuals with paraplegia and quadriplegia secondary to traumatic spinal cord injury classified as ASIA-A, ASIA-B, ASIA-C and ASIA-D from January 2013 to June 2014were involved in the study.

Inclusion criteria

18-60yr old male and female patients after 3 weeks of spinal cord injury attending outpatient and inpatient sections of Department of Physical Medicine and Rehabilitation, Emergency Department and Department of Spinal disorders of Christian Medical College Hospital, Vellore were included in this study after informed consent.

Exclusion criteria:

a. Patients with recent history (one week) of medical/surgical conditions which tend to raise the serum levels of ALP or CPK such as rhabdomyolysis, muscular dystrophy, myoscitis, myocardial infarction, myocarditis, hypothyroidism, severe orthopedic trauma, disorders of bile duct obstruction, celiac disease, muscle hematomas, or organ tear were excluded from the study.

b. Patients with existing ossification around hip joints/ bone pathology at the time of initial screening by pelvis X- ray were not included.

c. Individuals who have had hip fracture after the recruitment which is radiologically proven were excluded from the study.

d. Children and pregnant women were also excluded.

Selection of the study participants:

X-ray imaging was done on all the participants to rule out any preexisting ossification around hip joints. Baseline CPK & ALP and Osteocalcin levels were not taken into consideration for the recruitment of participants.

Sample size

A total of 43 patients with acute spinal cord injury within 3 weeks of duration, were assessed for eligibility to include in the study. 30 patients were recruited for the study and only 16 subjects could finish the study.

Methodology

ALP, CPK and Osteocalcin were measured at around 3^{rd}, 7^{th}, 11^{th} and 16^{th} week of the injury. Osteocalcin samples are stored under -70°C to prevent the wastage of the reagent kit till the end of the study. Base line hip x-ray was checked for every patient at the time of recruitment. All the blood samples were tested as per the standard operating procedures mentioned in the annexure. We interpreted the biomarker values as per the reference ranges established in CMCH Vellore Laboratory. The images of the reference standard i.e. Triple phase Technetium-99m bone scan were interpreted by nuclear medicine physician**.** The test results were not interpreted using clinical data from participants.

Laboratory Tests Information

CPK and ALP levels were measured using the Roche modular P800 analyzer with the respective reagents and N-mid osteocalcin by Roche modular E-170 analyzer. The Osteocalcin analyzer

was shown in Image no.1 and the reagent for osteocalcin which needs to be stored at 2-8°C which is shown in image no.2.

Image 1: N- MID Osteocalcin analyzer -Rosche Modular E-170

Image 2: N- MID Osteocalcin reagent.

CMCH, Vellore Normal ranges:

ALP (adult)	:	40-125 U/L
CPK Total Male	:	24-195U/L
Female	:	25-170 U/L

Osteocalcin by N-MID Elecsys Osteocalcin:

Women premenopausal	:	< 31.2ng/mL
Women postmenopausal	:	< 41.3ng/mL
Men> 50yrs	:	<26.3ng/mL
Men <50yrs	:	8-39ng/mL (with 2 SD; CMCH Vellore Laboratory)

Depending up on the bone scan results the participants were divided in to two groups. They were

1) Patients with HO and

2) Patients without HO.

The methodology of the study is shown briefly in the figure no.1

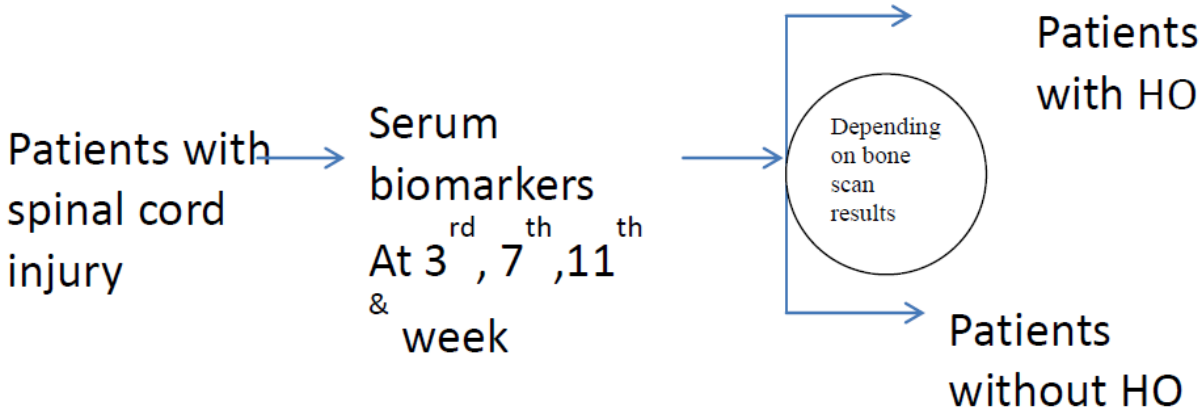


Figure 1. Methodology of the current study

RESULTS

RESULTS

30 patients were screened with pelvis X-ray and recruited for the current study. There were 14 drop outs during the 16 week follow up period and 16 completed the study with the establishment of the diagnosis by triple phase bone scan as shown in Figure 2.

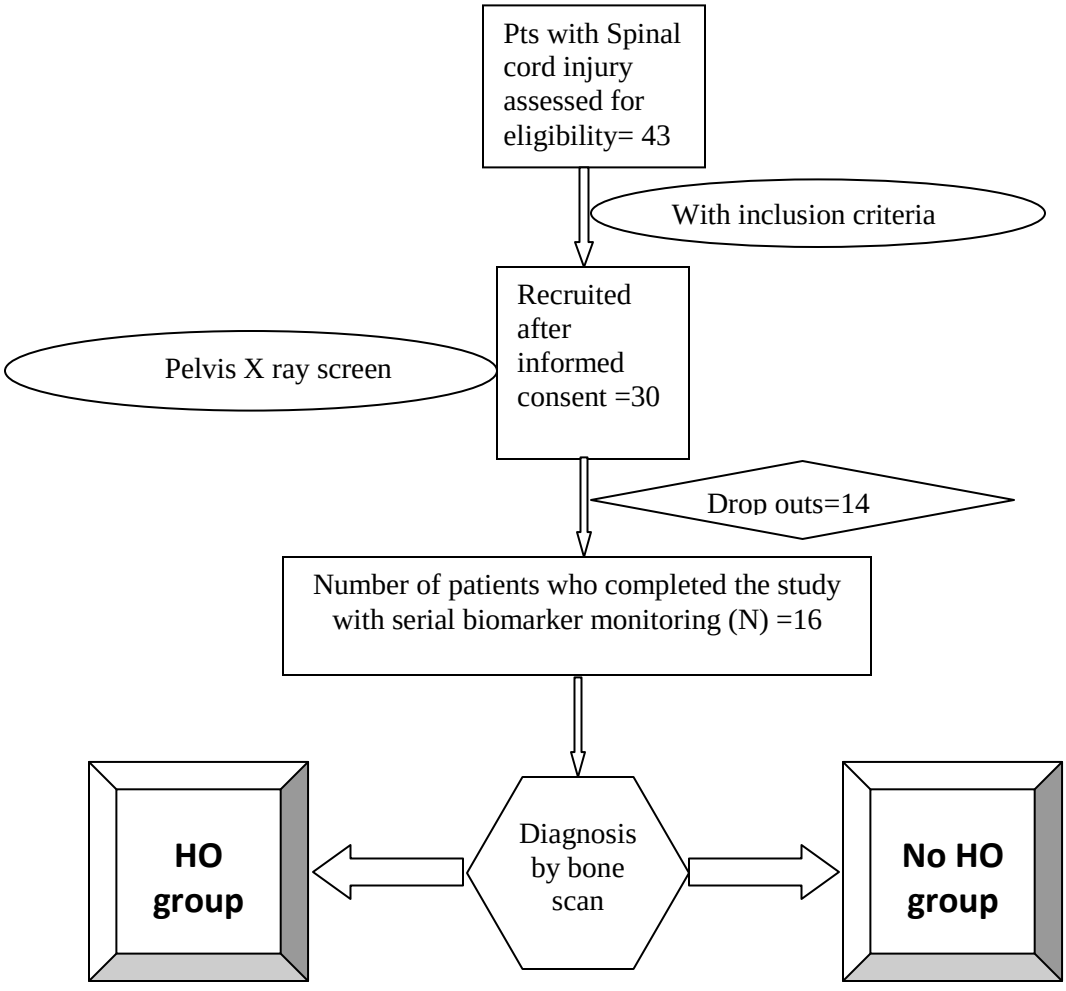


Figure 2. Flow chart depicting the participants of the study

Demography of the study participants

Age group distribution

Age in yrs	Number of Patients with SCI	Percentage
18-20	2	12.50
21-30	9	56.25
31-40	2	12.50
41-50	1	06.25
51-60	2	12.50
Total	16	100

Table 4. Distribution of patients according to age group

Out of the 16 study patients 9 patients were from the age group of 21-30. This is consistent with most of the spinal cord injury incidence studies. The mean of the ages of the participants is 32.6 yrs.

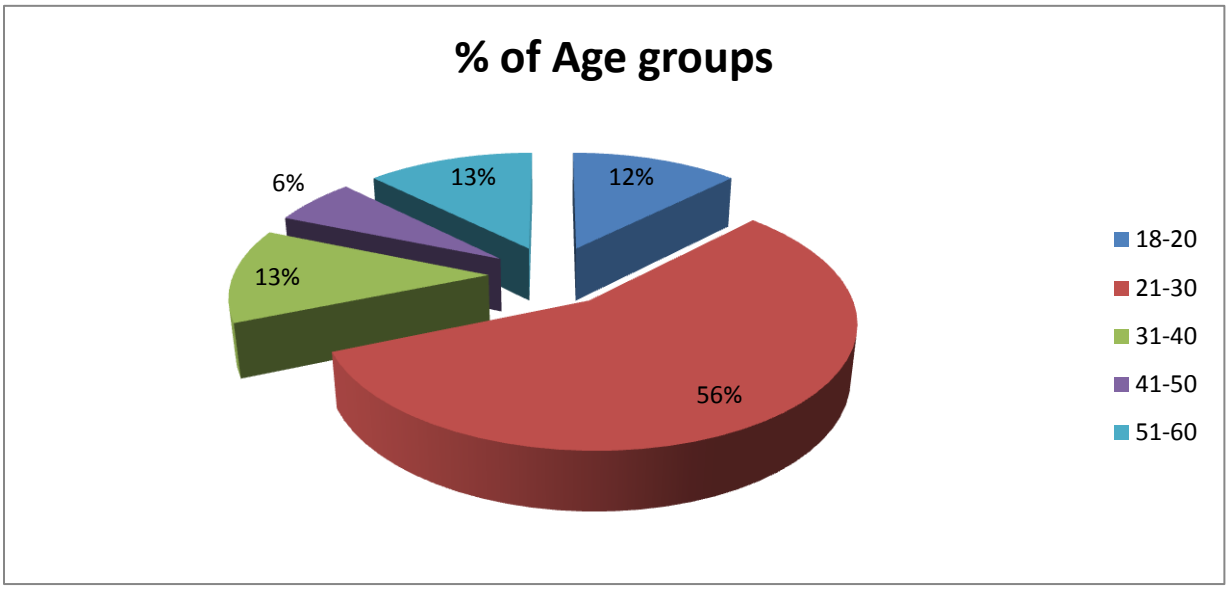


Figure 3.Distribution of patients according to age group

Gender distribution

Gender	Number	Percentage
Males	15	94
Female	1	6

Table 5. Distribution of patients according to group

Only one female patient was recruited in our study. This is in line with most of the studies showing less incidence of spinal cord injury in females.

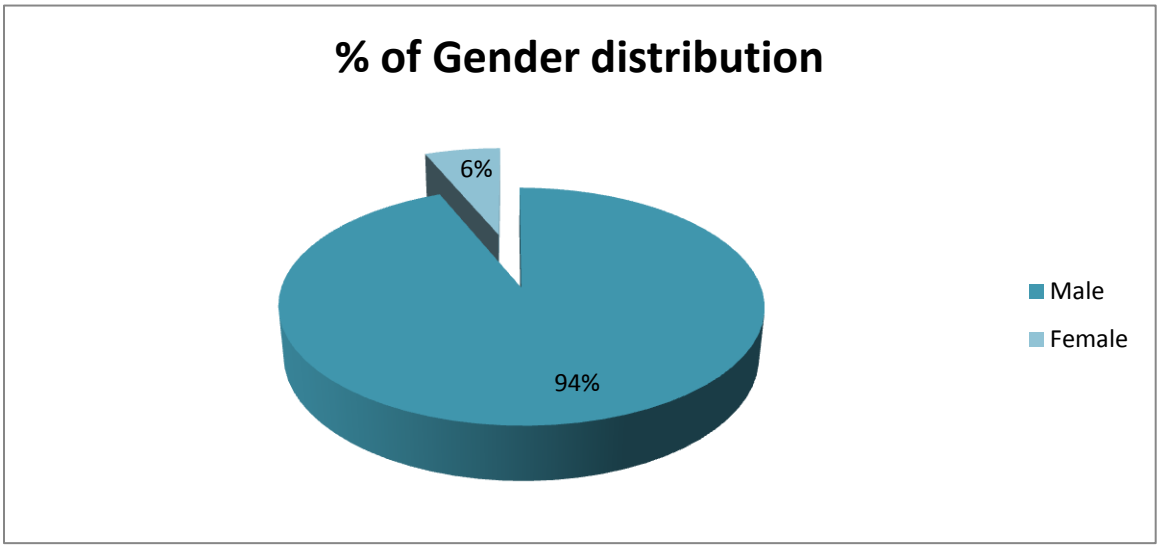


Figure 4.Distribution of patients according to gender

Distribution according to mode of injury

Mode of injury	Number	Percentage
Fall	11	68.75
RTA	5	31.25

RTA: Road traffic accidents

Table 6. Distribution of patients according to mode of injury

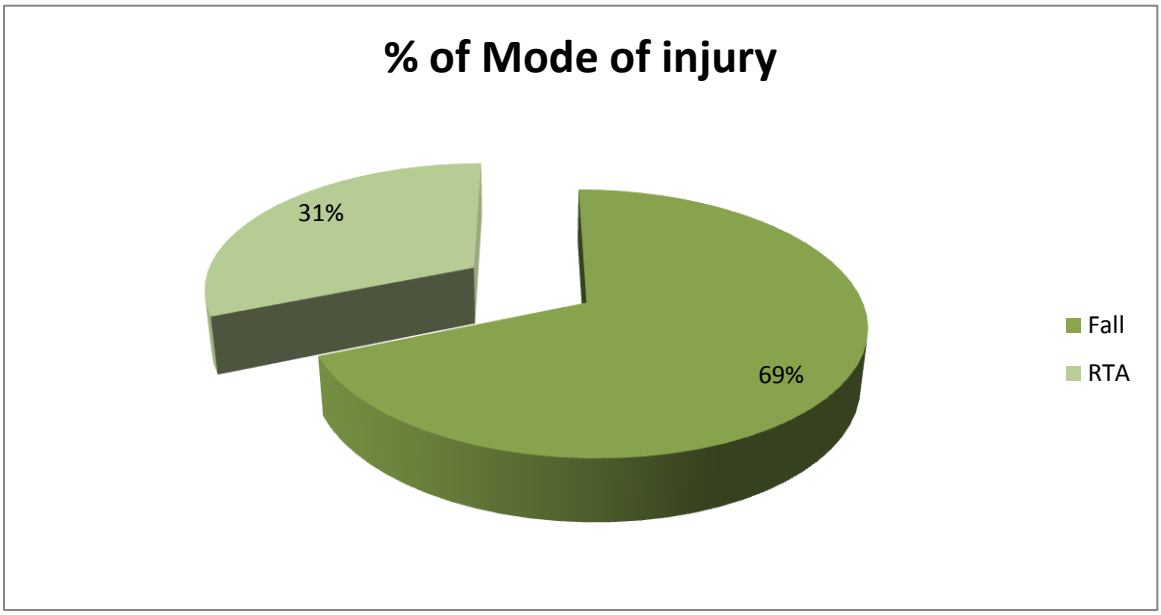


Figure 5.Distribution of patients according to mode of injury

Distribution according to completeness of the spinal cord injury

	Number of patients	Percentage
Complete	11	68.75
Incomplete	5	31.25

Table 7. Distribution of patients according to complete/ incomplete

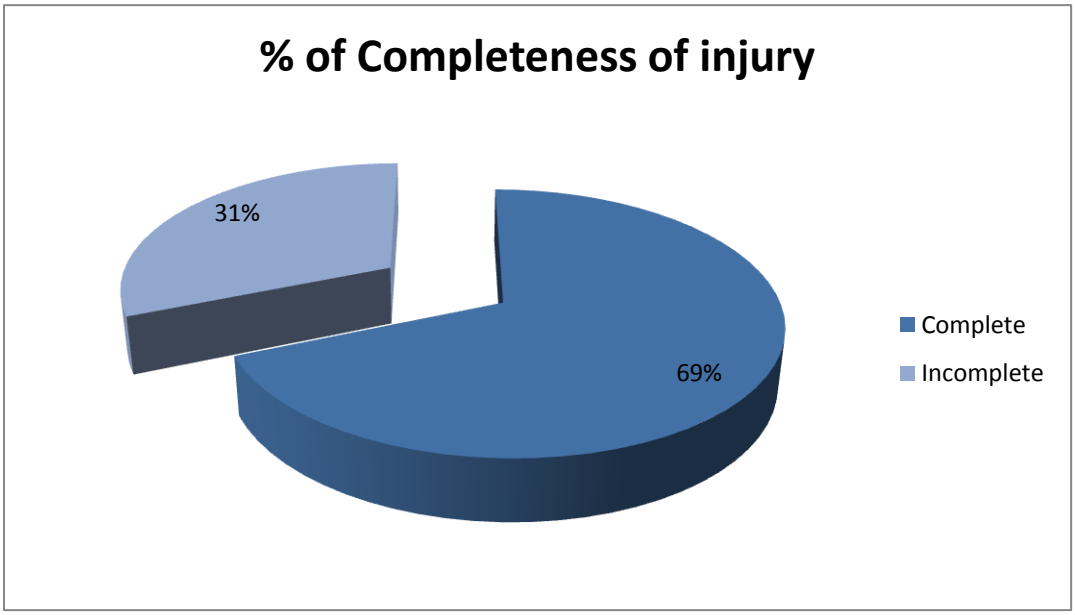


Figure 6.Distribution of patients according to complete/ incomplete

Distribution according to type of management

Management	Number of patients	Percentage
Conservative	2	12.5
Surgical	14	87.5

Table 8. Distribution of patients according to type of management

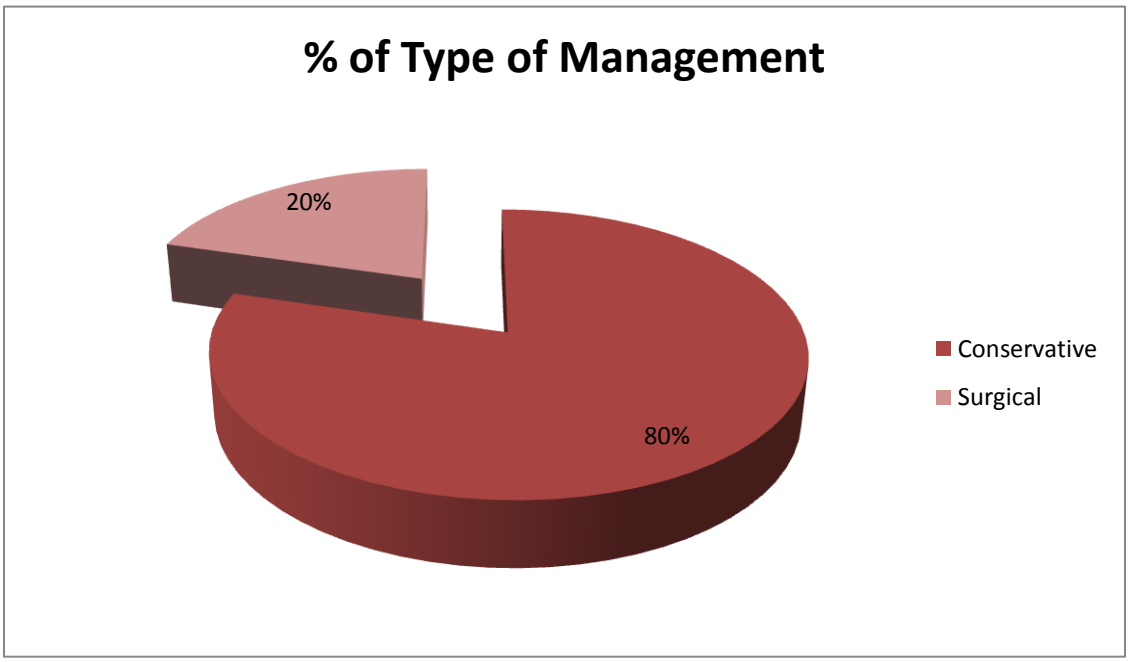


Figure 7.Distribution of patients according to type of management

Distribution according to presence of HO

	Number	Percentage
HO	4	25
No HO	12	75
Total	16	100

Table 9. Distribution of patients according to presence of HO

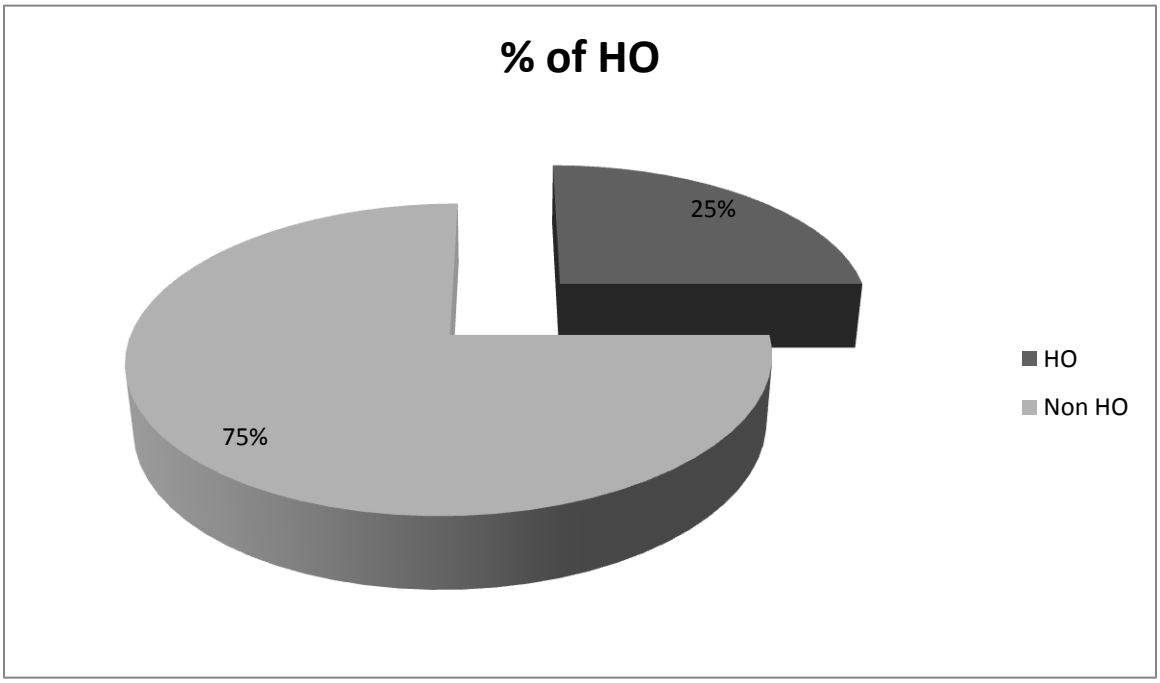
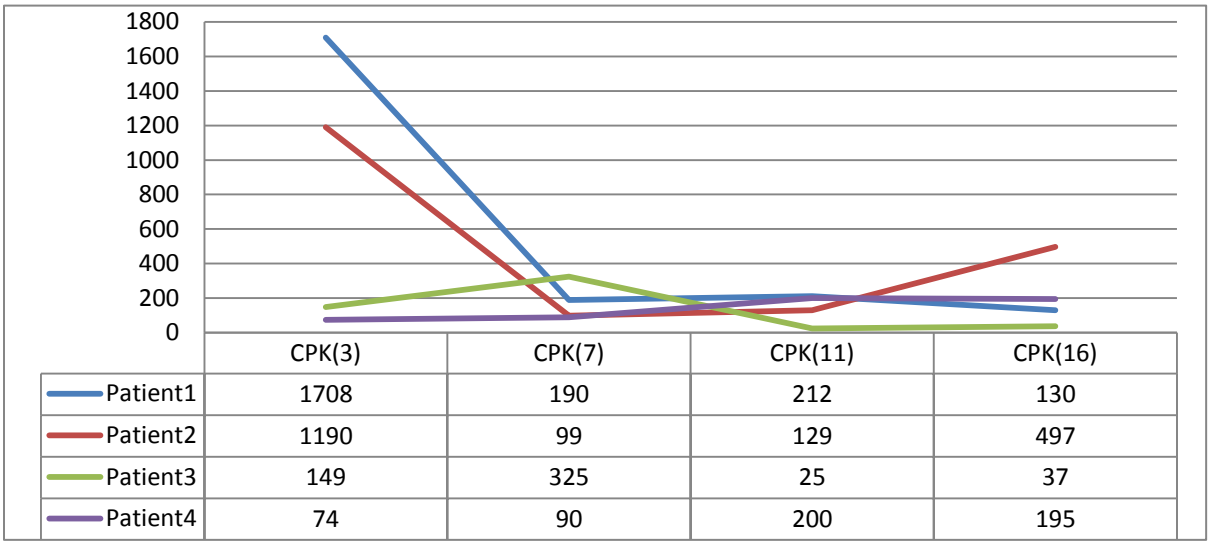


Figure 8. Percentage of SCI patients that developed HO

Individual biomarker levels in HO group patients during the initial 16weeks



	CPK(3)	CPK(7)	CPK(11)	CPK(16)
Patient1	1708	190	212	130
Patient2	1190	99	129	497
Patient3	149	325	25	37
Patient4	74	90	200	195

Figure 9. In HO group patients, the trend of CPK levels during the initial 16weeks showing heterogeneity. X axis (weeks); Y axis- CPK levels in Units per litre.

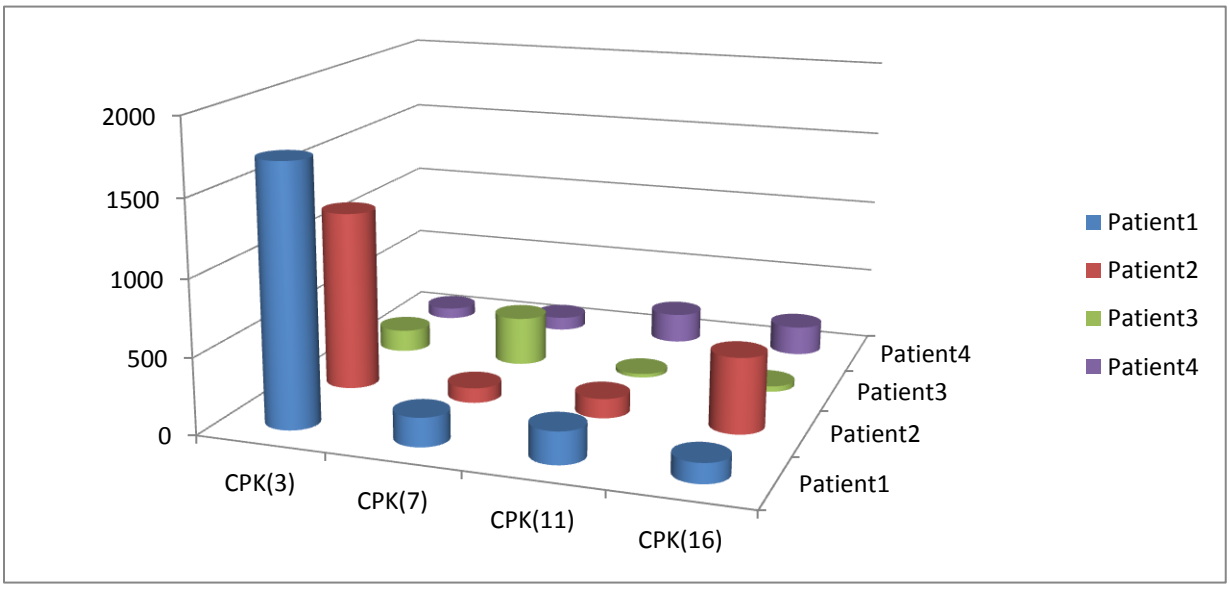
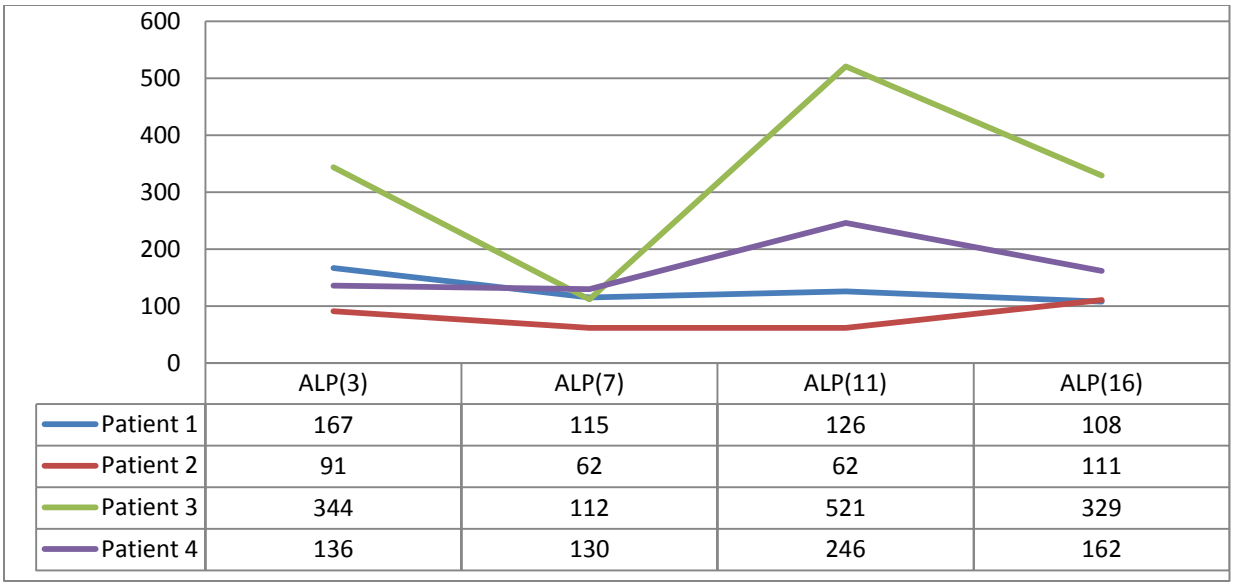


Figure 10. CPK patterns in the sera of four patients who developed HO. X axis (weeks); Y axis- CPK levels in Units per litre.



	ALP(3)	ALP(7)	ALP(11)	ALP(16)
Patient 1	167	115	126	108
Patient 2	91	62	62	111
Patient 3	344	112	521	329
Patient 4	136	130	246	162

Figure 11. In HO group patients, the trend of ALP levels during the initial 16weeks. X axis (weeks); Y axis- ALP levels in Units per litre.

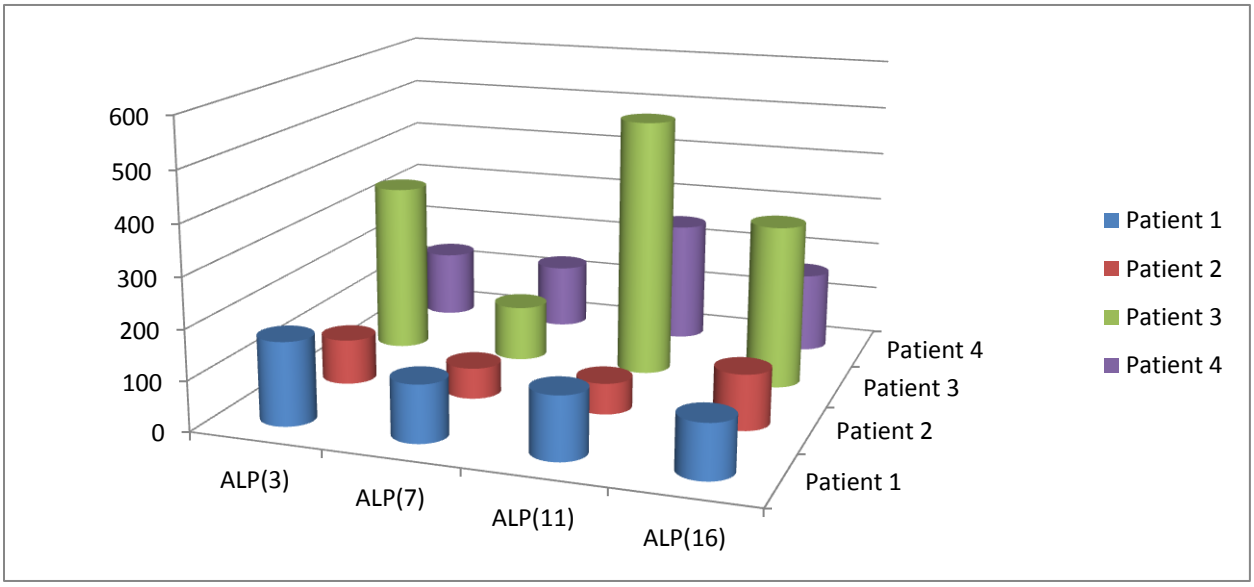
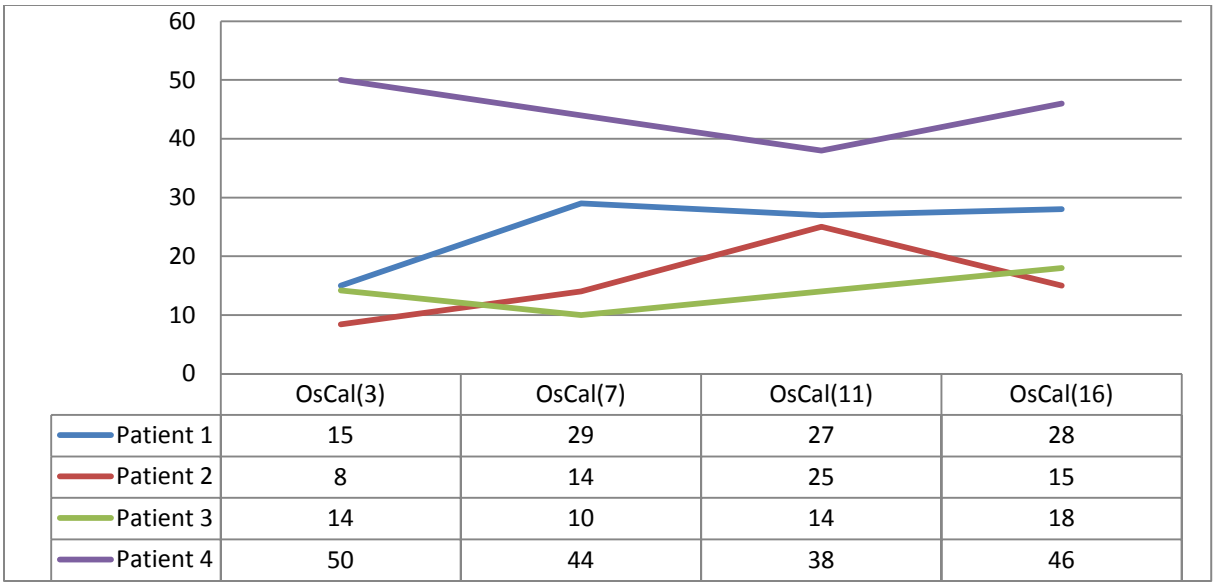


Figure 12. ALP patterns in the sera of four patients who developed HO. X axis (weeks); Y axis- ALP levels in Units per litre.



	OsCal(3)	OsCal(7)	OsCal(11)	OsCal(16)
Patient 1	15	29	27	28
Patient 2	8	14	25	15
Patient 3	14	10	14	18
Patient 4	50	44	38	46

Figure 13. In HO group patients, the trend of osteocalcin levels during the initial 16weeks. X axis (weeks); Y axis- Osteocalcin levels in nanograms per millilitre.

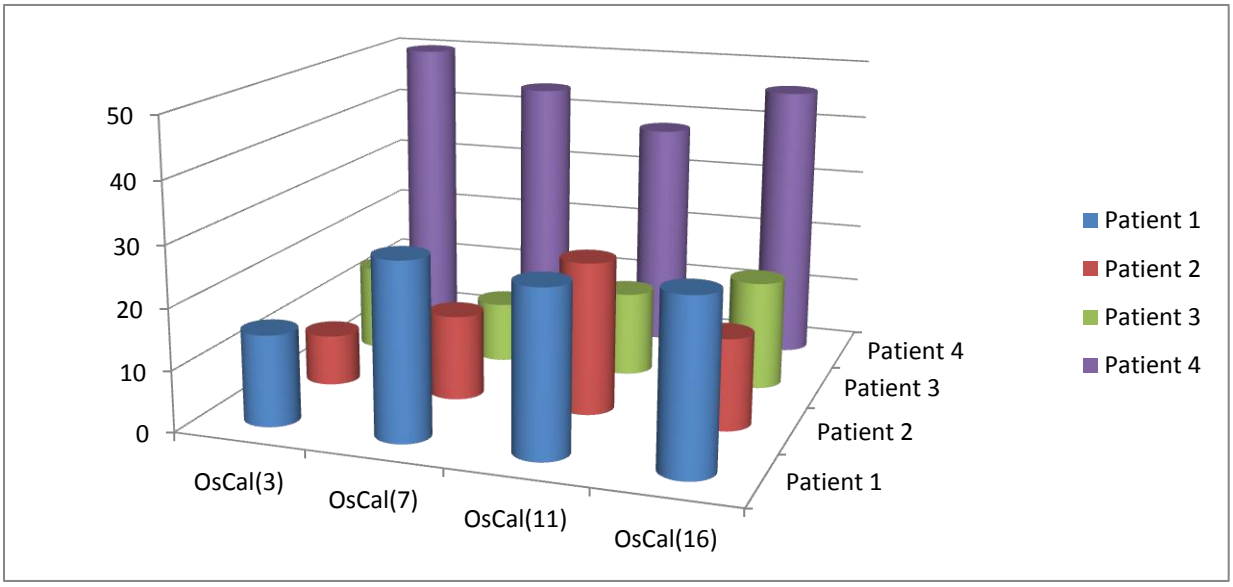


Figure 14. Osteocalcin patterns in the sera of four patients who developed HO. X axis (weeks); Y axis- Osteocalcin levels in nanograms per millilitre.

Individual biomarker levels in non-HO group patients during the initial 16weeks

S.No	CPK(3)	CPK(7)	CPK(11)	CPK(16)
Patient 1	314	96	72	58
Patient 2	990	95	93	160
Patient 3	418	207	116	90
Patient 4	179	79	124	92
Patient 5	82	47	93	98
Patient 6	390	71	70	85
Patient 7	43	42	49	65
Patient 8	352	117	78	74
Patient 9	136	423	273	195
Patient 10	304	446	226	119
Patient 11	270	44	66	47
Patient 12	228	40	52	36

Table 10. CPK levels at different weeks in patients belonging to non-HO group

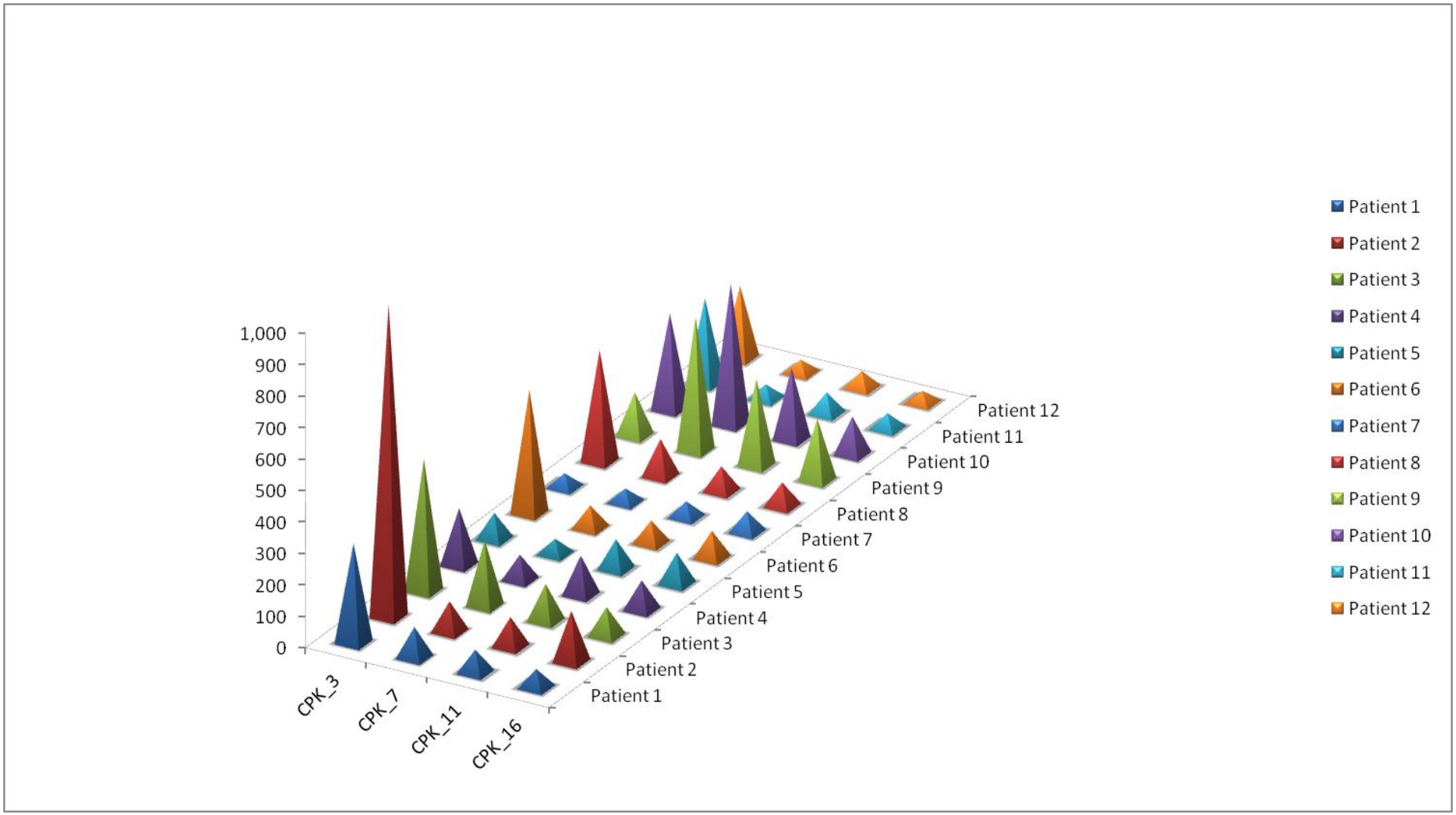


Figure.15 CPK levels at different weeks in patients belonging to non-HO group

S.No	ALP(3)	ALP(7)	ALP(11)	ALP(16)
Patient 1	109	70	87	77
Patient 2	133	111	95	84
Patient 3	106	111	127	169
Patient 4	98	77	89	77
Patient 5	87	61	69	66
Patient 6	212	79	85	94
Patient 7	345	184	138	124
Patient 8	155	134	127	113
Patient 9	119	99	78	72
Patient 10	156	110	106	107
Patient 11	159	141	88	80
Patient 12	184	93	79	116

Table 11. ALP levels at different weeks in patients belonging to non-HO group

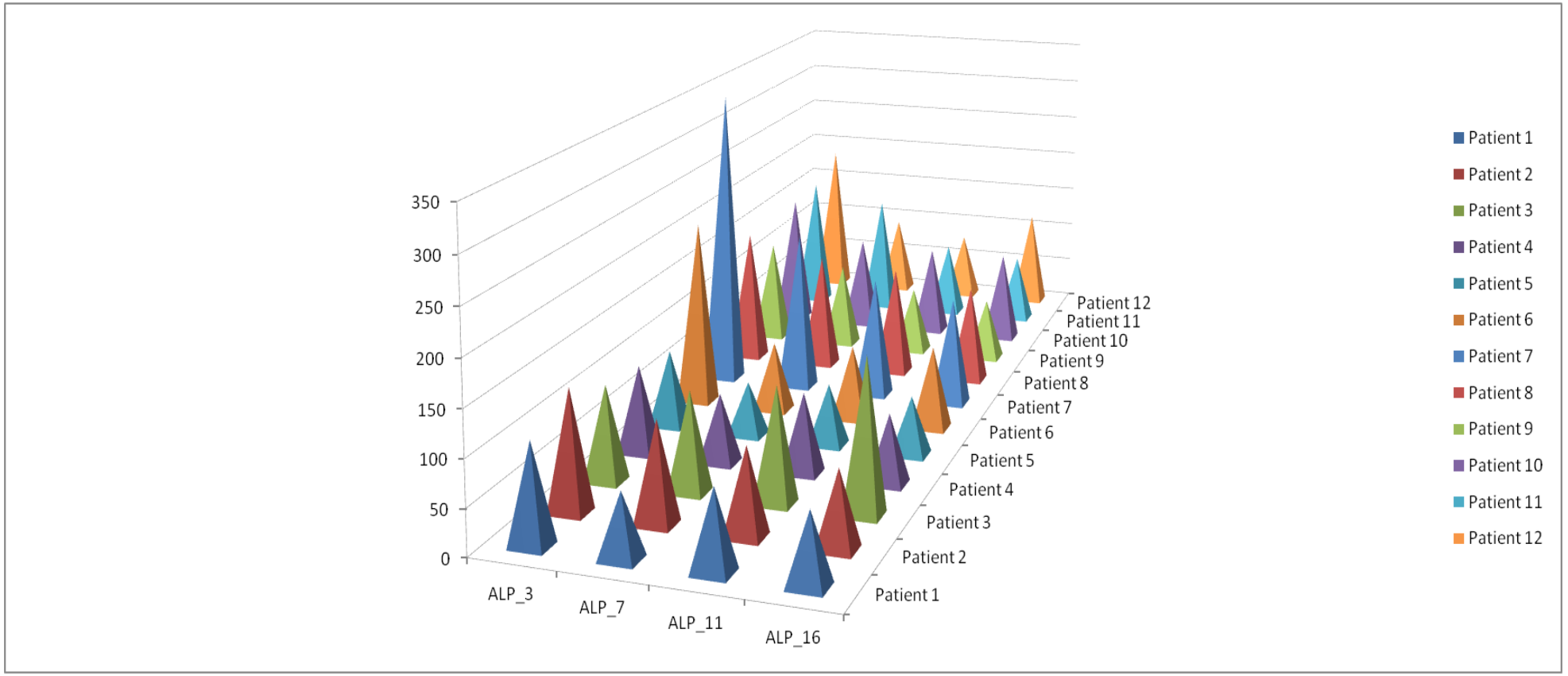


Figure.16 ALP levels at different weeks in patients belonging to non-HO group

S.No	OsCal(3)	OsCal(7)	OsCal(11)	OsCal(16)
Patient 1	14	9	18	22
Patient 2	10	25	21	-
Patient 3	43	38	35	39
Patient 4	18	13	21	22
Patient 5	9	21	16	13
Patient 6	11	24	31	28
Patient 7	14	21	30	30
Patient 8	18	28	27	32
Patient 9	15	21	26	15
Patient 10	26	22	37	44
Patient 11	18	32	30	27
Patient 12	6	18	17	13

Table 12. Osteocalcin levels at different weeks in patients belonging to non-HO group

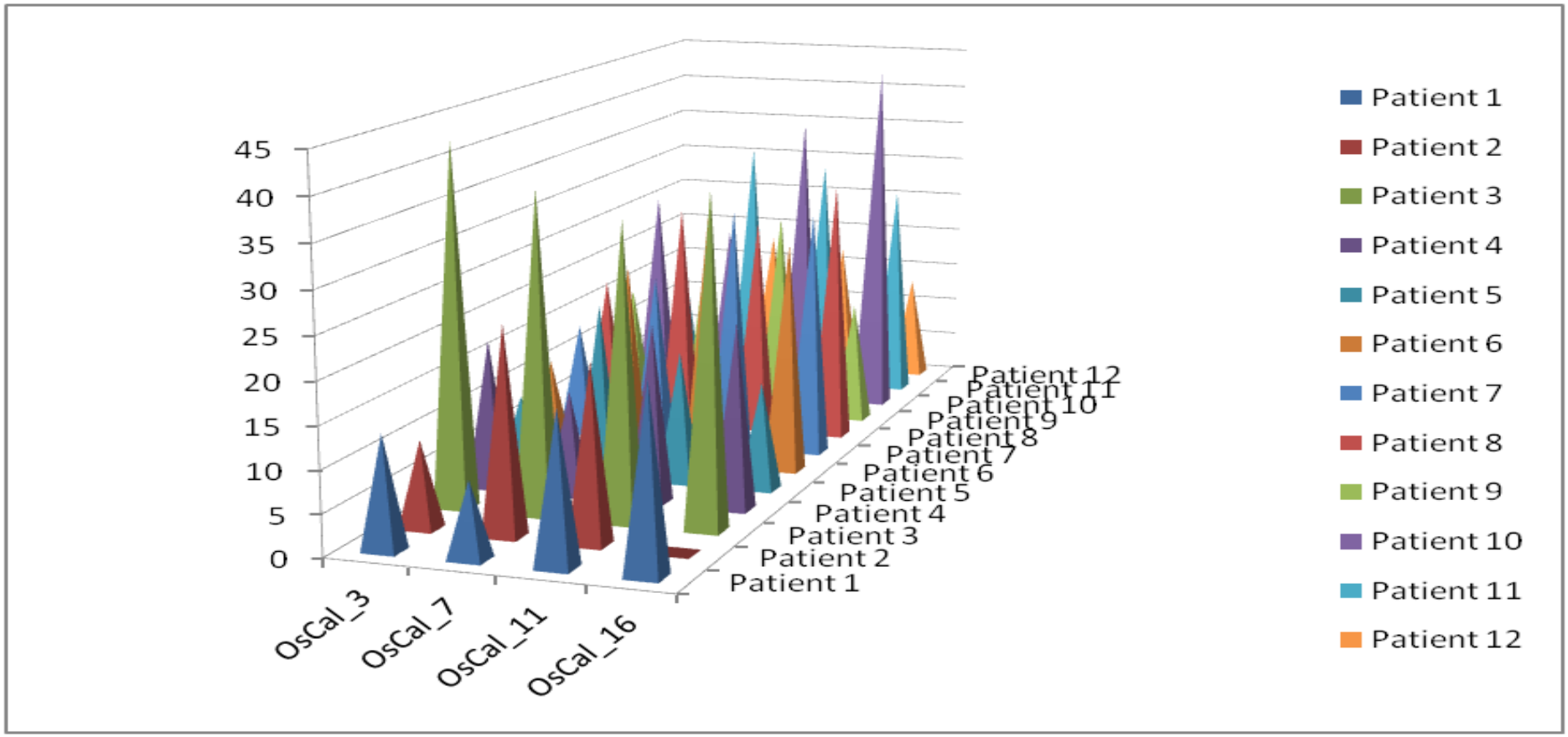


Figure.17 Osteocalcin levels at different weeks in patients belonging to non-HO group

Figure 18. Initial pelvis X- ray of patient no.1 shows normal bilateral hip joints

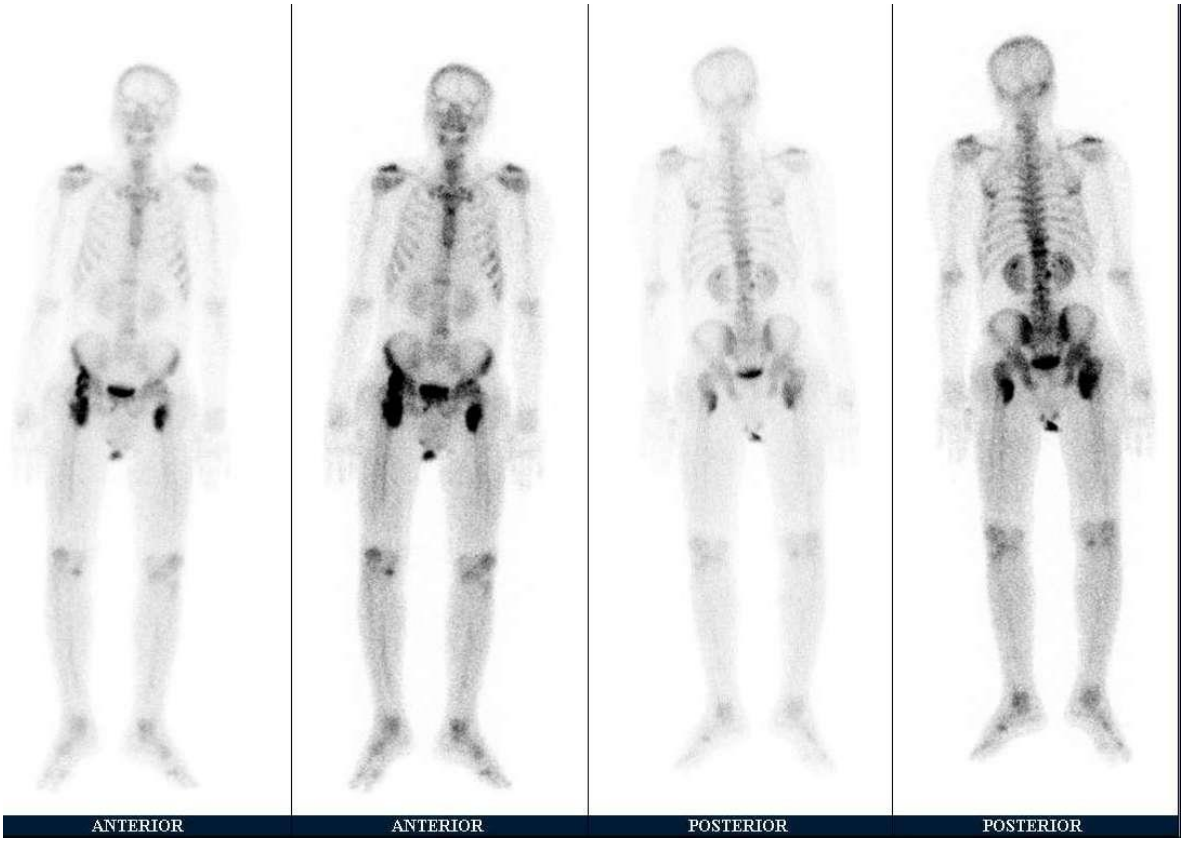


Figure 19. Active HO around bilateral hip joints & proximal femurs in bone scan at 16 weeks in patient no.1

Figure 20. Initial pelvis X- ray of patient no.2 shows normal bilateral hip joints

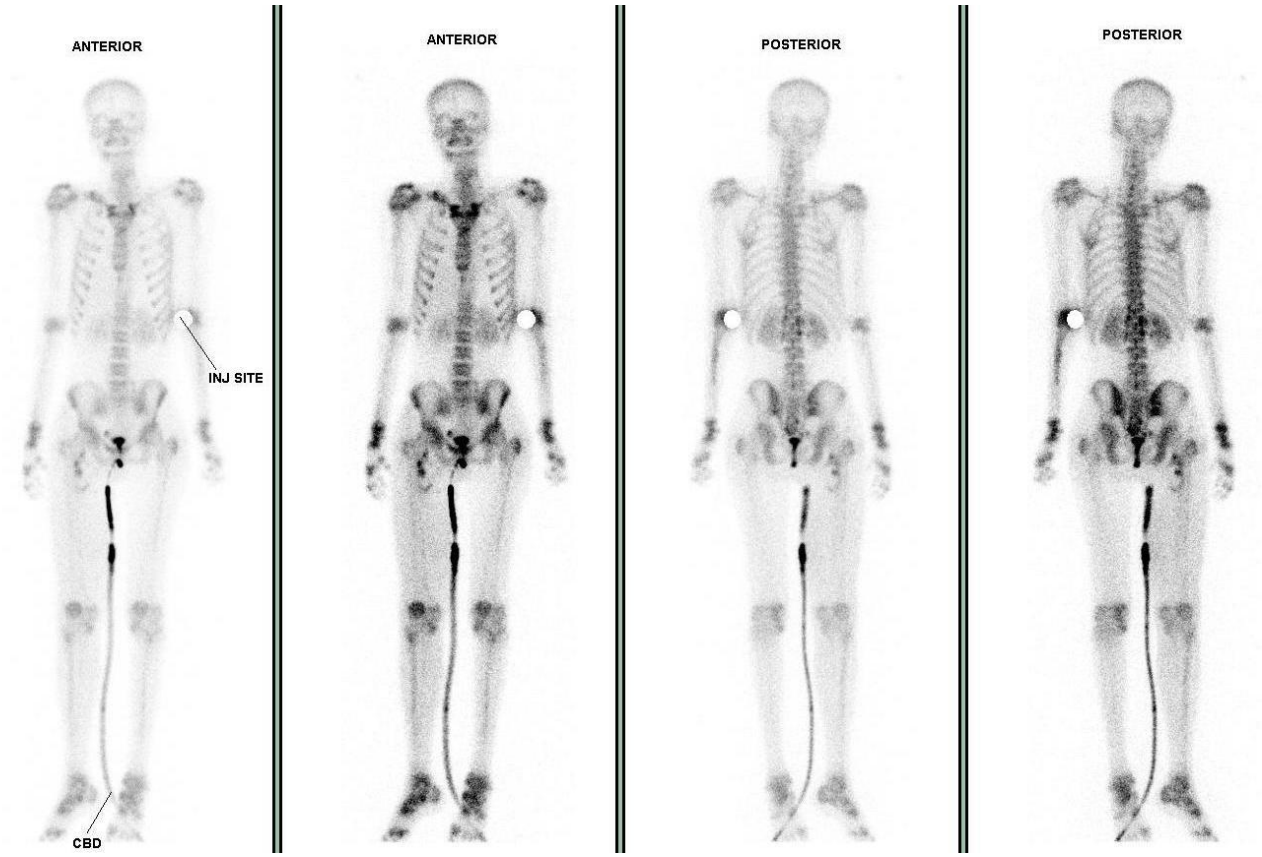


Figure 21. Active HO in bilateral hip joints in bone scan at 16 weeks in patient no.2

Figure 22. Initial pelvis X- ray of patient no.3 shows normal bilateral hip joints

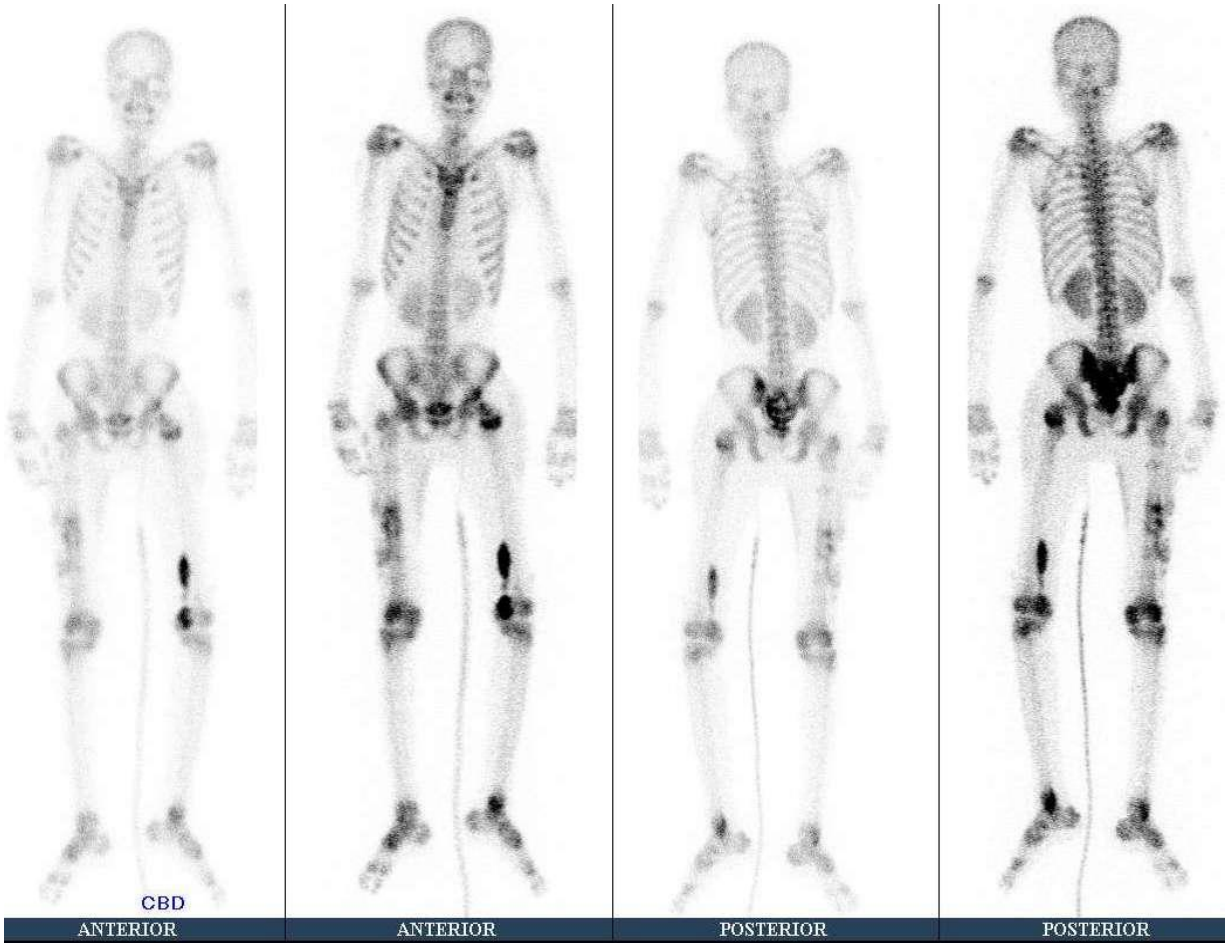


Figure 23. HO in pelvis and right thigh in bone scan at 16 weeks in patient no.3

Figure 24. Initial pelvis X- ray of patient no.4 shows normal bilateral hip joints

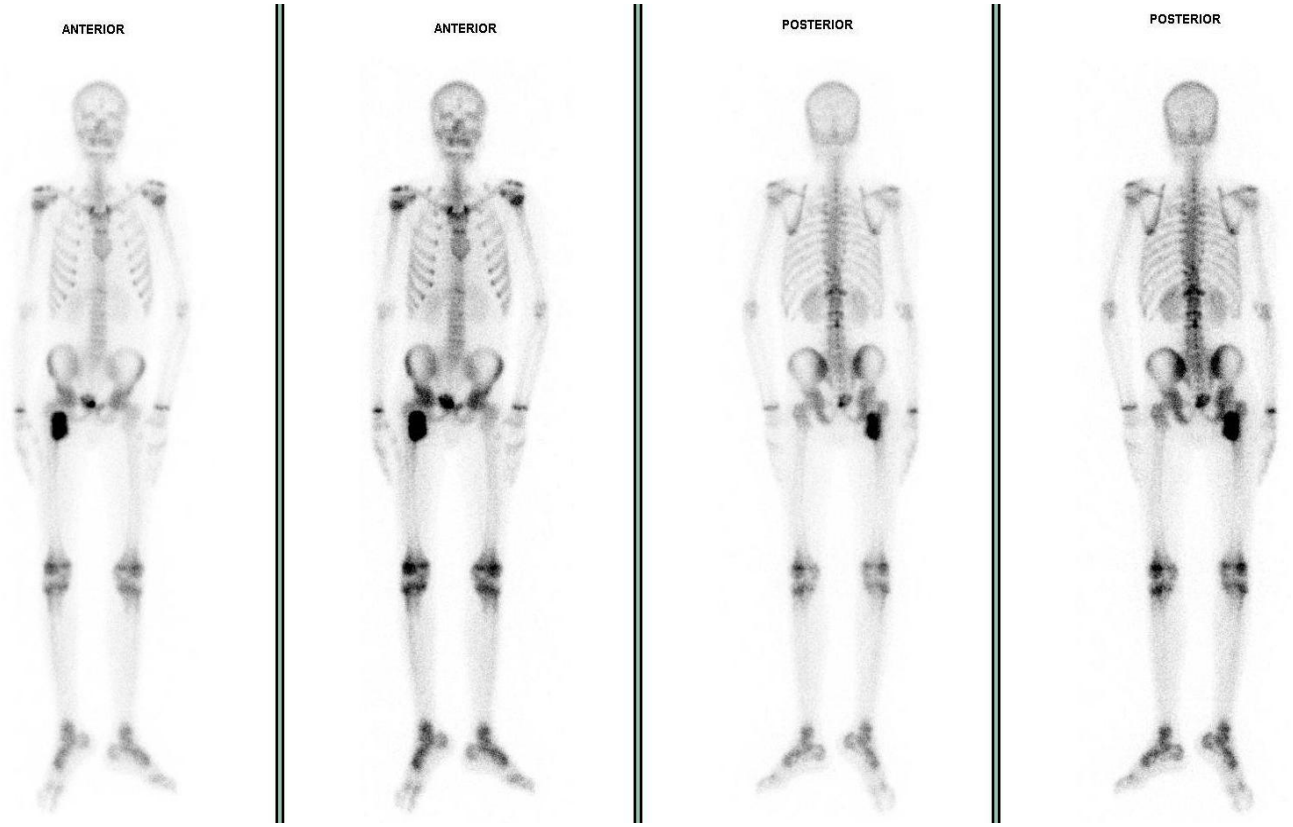


Figure 25. Active HO of left proximal femur in bone scan at 16 weeks in patient no.4

STATISTICAL ANALYSIS

Statistical Analysis

The final results were compared and analyzed with two sided Fisher Exact test using SPSS software. We found there is no significant correlation of CPK values at different weeks with the later developing heterotopic ossification in the four patients and ALP values at different weeks showed there is no specific co relation with the HO that was noticed after 16weeks.

CPK at 3weeks

The range of CPK levels in HO patients at 3rd week is between 74 and 1.708 U/L with mean value of 780U/L. The Sensitivity and specificity of the third week CPK is calculated from the following two way tables. The *P* value for CPK at 3rd week in diagnosing HO is 0.547 which is not significant.

CPK at 3rd week	HO	No HO	Total
Positive	2	9	11
Negative	2	3	5
Total	4	12	16

Sensitivity = 50% Specificity = 25% PPV = 18.18% NPV = 60%

CPK at 7 weeks

The range of CPK levels in HO patients at 7th week is between 90 and 325 U/L with mean value of 176U/L. The Sensitivity and specificity of the seventh week CPK is calculated from the following two way tables. The *P* value of CPK at 7th week in diagnosing HO is 1.0 which is not significant.

CPK at 7th week	HO	No HO	Total
Positive	1	3	4
Negative	3	9	12
Total	4	12	16

Sensitivity = 25% Specificity =75 %

PPV = 25% NPV = 75%

CPK at 11 weeks

The range of CPK levels in HO patients at 11th week is between 25 and 212 U/L with mean value of 142U/L. The Sensitivity and specificity of the eleventh week CPK is calculated from the following two way tables. The *P* value of CPK at 11th week in diagnosing HO is 0.245 which is not significant.

CPK at 11th week	HO	No HO	Total
Positive	2	2	4
Negative	2	10	12
Total	4	12	16

Sensitivity =50 % Specificity =83.33%

PPV = 50% NPV = 83.33%

CPK at 16 weeks

The range of CPK levels in HO patients at 16th week is between 37and 497 U/L with mean value of 215U/L. The Sensitivity and specificity of the sixteenth week CPK is calculated from the following two way tables. The *P* value of CPK at 16th week in diagnosing HO is 0.250 which is not significant.

CPK at 16th week	HO	No HO	Total
Positive	1	0	1
Negative	3	12	15
Total	4	12	16

Sensitivity = 25% Specificity = 1%

PPV = 1% NPV = 80%

ALP at 3 weeks

The range of ALP levels in HO patients at 3rd week is between 91 and 344 U/L with mean value of 185U/L. The Sensitivity and specificity of the third week ALP is calculated from the following two way tables. The *P* value of ALP at 3rd week in diagnosing HO is 1.0 which is not significant.

ALP at 3rd week	HO	No HO	Total
Positive	3	8	11
Negative	1	4	5
Total	4	12	16

Sensitivity = 75% Specificity = 33.33%

PPV = 27.27% NPV = 80%

ALP at 7 weeks

The range of ALP levels in HO patients at 7th week is between 62 and 130 U/L with mean value of 105U/L. The Sensitivity and specificity of the seventh week ALP is calculated from the following two way tables. The *P* value of ALP at 7th week in diagnosing HO is 1.0 which is not significant.

ALP at 7th week	HO	No HO	Total
Positive	1	3	4
Negative	3	9	11
Total	4	12	16

Sensitivity = 25% Specificity = 75%

PPV = 25% NPV = 75%

ALP at 11 weeks

The range of ALP levels in HO patients at 11th week is between 62 and 521 U/L with mean value of 239U/L. The Sensitivity and specificity of the eleventh week ALP is calculated from the following two way tables. The *P* value of ALP at 11th week in diagnosing HO is 0.118 which is not significant.

ALP at 11th week	HO	No HO	Total
Positive	3	3	6
Negative	1	9	10
Total	4	12	16

Sensitivity = 75% Specificity = 75%

PPV = 50% NPV = 90%

ALP at 16 weeks

The range of ALP levels in HO patients at 16th week is between 108 and 329U/L with mean value of 178U/L. The Sensitivity and specificity of the sixteenth week ALP is calculated from the following two way tables. The *P* value of ALP at 16th week in diagnosing HO is 0.136 which is not significant.

ALP at 16th week	HO	No HO	Total
Positive	2	1	3
Negative	2	11	13
Total	4	12	16

Sensitivity = 50% Specificity = 91.66%

PPV = 66,66% NPV = 84.61%

Osteocalcin at 3 weeks

The range of Osteocalcin levels in HO patients at 3rd week is between 8 and 50ng/dL with mean value of 22ng/dL. The Sensitivity and specificity of the third week osteocalcin is calculated from the following two way tables. The *P* value of Osteocalcin at 3rd week in diagnosing HO is 0.450 which is not significant.

Osteocalcin at 3rd week	HO	No HO	Total
Positive	1	1	2
Negative	3	11	14
Total	4	12	16

Sensitivity = 25% Specificity = 91.66%

PPV = 50% NPV = 78.57%

Osteocalcin at 7 weeks

The range of Osteocalcin levels in HO patients at 7th week is between 10 and 44ng/dL with mean value of 24ng/dL. The Sensitivity and specificity of the seventh week osteocalcin is calculated from the following two way tables. The *P* value of Osteocalcin at 7th week in diagnosing HO is 0.050.

Osteocalcin at 7th week	HO	No HO	Total
Positive	2	0	2
Negative	2	12	14
Total	4	12	16

Sensitivity = 50% Specificity = 100%

PPV = 100% NPV = 85.71%

Osteocalcin at 11 weeks

The range of Osteocalcin levels in HO patients at 11th week is between 14 and 38ng/dL with mean value of 26ng/dL. The Sensitivity and specificity of the eleventh week osteocalcin is calculated from the following two way tables. The *P* value of Osteocalcin at 11th week in diagnosing HO is 0.250 which is not significant.

Osteocalcin at 11th week	HO	No HO	Total
Positive	1	0	1
Negative	3	12	15
Total	4	12	16

Sensitivity = 25% Specificity = 100%

PPV = 100% NPV = 80%

Osteocalcin at 16 weeks

The range of Osteocalcin levels in HO patients at 16th week is between 15 and 46ng/dL with mean value of 27ng/dL. The Sensitivity and specificity of the sixteenth week osteocalcin is calculated from the following two way tables. We were not able to measure the osteocalcin level at sixteenth week for one patient in the no HO group. The *P* value of Osteocalcin at 16th week in diagnosing HO is 0.154 which is not significant.

Osteocalcin at 16th week	HO	No HO	Total
Positive	2	1	3
Negative	2	10	12
Total	4	11	15

Sensitivity = 50% Specificity = 90.9%

PPV = 66.66% NPV = 83.33%

The Sensitivity, specificity, PPV and NPV of CPK levels at different weeks are as follows

	CPK(3)	CPK(7)	CPK(11)	CPK(16)
Sensitivity	50	25	50	25
Specificity	25	75	83.33	1
PPV	18.18	25	50	1
NPV	60	75	83.33	80

Table 13. Sensitivity, specificity, PPV and NPV of CPK levels at different weeks

The Sensitivity, specificity, PPV and NPV of ALP levels at different weeks are as follows

	ALP(3)%	ALP (7)%	ALP (11)%	ALP (16)%
Sensitivity	75	25	75	50
Specificity	33.33	75	75	91.66
PPV	27.27	25	50	66.66
NPV	80	75	90	84.61

Table 14. Sensitivity, specificity, PPV and NPV of ALP levels at different weeks

The Sensitivity, specificity, PPV and NPV of Osteocalcin levels at different weeks are as follows

	Osteocalcin(3)%	Osteocalcin(7)% *P* Value- 0.050	Osteocalcin(11)% *P* value-0.250	Osteocalcin(16)%
Sensitivity(%)	25	50	25	50
Specificity	91.66	100	100	90.90
PPV	50	50	100	66.66
NPV	78.57	85.71	80	83.33

Table 15. Sensitivity, specificity, PPV and NPV of Osteocalcin levels at different weeks

DISCUSSION

DISCUSSION

The necessity of a test, which is low cost and quick yet reliable, for early diagnosis of HO and initiation of prompt treatment has been a biggest question since the description of the condition in the literature. In this study we attempted to see the diagnostic value of various biochemical markers like CPK, ALP and Osteocalcin.

Creatine phosphokinase:

Identifying the role of CPK in diagnosis of later forming HO has been a greater challenge for researchers. In 2003, *Singh et al* observed the greater predictive value of CPK for subsequent HO formation compared to elevated ALP levels(22). They noticed a correlation between high levels of CPK and grades 2-4 of *Brooker* classification of HO. That initiated more focus on CPK as a potential tool in further studies. We, in our study attempted to see the behavior of CPK levels in patients that developed HO. As the peak period of HO is seen in early four months in spinal cord injury patients, we diagnosed the condition with the gold standard test, the triple phase bone scan at the end of study. Out of four patients who developed HO, two had increased levels of CPK levels at 3rd week which is not statistically significant. Out of these two, only one patient had massive HO with ankylosis though HO grading and quantification were not done using the plain X-rays. Interestingly both these patients had normal levels of CPK after 1month duration. Hence checking the CPK levels might be still remaining as inconclusive and may not be useful indicator for the subsequent HO development. This could be conflicting with the previously published data with almost same numbers but with relatively more number of HO patients i.e. 7 vs. 4. Few

unidentified conditions like muscle injury, subtle liver laceration which is asymptomatic and surgical handling of the muscle tissues can influence the levels of CPK.

Alkaline phosphatase:

ALP has been the test of choice for monitoring the growth of HO as this represents the osteoblastic activity. In the present study, we observed all the four subjects who developed HO had vertebral fracture and were all managed surgically. The rising trend of ALP in developing HO has been well described in the literature. We did not observe that pattern in our study in all the four patients who developed HO. Only one patient out of these had persistent elevated values. This could be due to the timing of maturity of HO which influences the ALP values. This particular point of time can only be identified through serial bone scans which are not economically feasible. As all these patients had fracture of vertebrae, commenting on the ALP elevation pattern may not be of much significance.

In the initial four months, the ALP levels are affected by the associated fractures and surgical intervention. Most of the patients with complete spinal cord injuries usually have concomitant limb bone fractures or at least vertebral fractures. *Cassar Pullicino* noticed the rising ALP trend, (though the values are still within normal limits) may be present in patients who are diagnosed with bone scan. But we could not see any rising trend as such in the patients with HO. Another pitfall of ALP is, minimal HO may not be evident. This we observed in one of four patients with HO in whom there is no single elevated ALP value observed. Hence one should not rely on serum ALP values especially during the first four months.

Osteocalcin:

We failed to find any research work done in prediction of the HO in spinal cord injury patients. A study done in traumatic brain injury patients in 1993 showed osteocalcin as a poor adjunct to confirm the diagnosis and it did not show any maturation of the condition. The basic pathophysiology in HO in spinal cord injury patients and brain injury patients could be potentially different as it is obvious from one study that the fracture healing in brain injury is more than that of without associated brain injury(52). In our study, we noticed osteocalcin at 7^{th} week could be a possible test to identify possibility of not developing HO if the test is normal. (Sensitivity is 100% and Negative predictive value NPV = 85.71%, *P* Value 0.050). But the actual significance should be checked with the larger sample sizes. Just like other biomarkers, Osteocalcin also has peculiar way of its presence in the blood. During the early phase of mineralization after fracture, osteocalcin levels will be high and as the mineralization continues the levels will reduce(96). Our study substantiates the earlier finding in brain injury patients, that it may not be a useful tool in diagnosis of the condition and cannot imply the maturity of the same.

CONCLUSIONS

Conclusions

1. CPK may not be a good early biochemical marker for diagnosing the HO in spinal cord injury patients. The rising trend may not be observed in all patients who develop HO implying its poor quality to assess the HO maturity.
2. ALP can be serially monitored to check the osteoblastic activity only in the context of absence of bone injury. Interpretation of ALP values in first four months of the injury should be done carefully.
3. Osteocalcin in spinal cord injury patients is not a good tool in predicting the future developing HO.

LIMITATIONS OF THE STUDY

Limitations of the Study

1. Sample size is small to conclude firmly on any of the biomarkers to predict the HO. The unequal distribution of the patients in both HO and No HO groups may be another confounding factor.
2. The dropout rates are high in the current study. We noticed less enthusiasm of the participants as it is economically burden for most of them after a major incident of spinal cord injury.
3. Clinical data especially range of hip joints were not accurately assessed at different intervals. Hence it was not analysed in the study which could have given valid functional information.

FURTHER RESEARCH

RECOMMENDATIONS

Further Research Recommendations

1. Influence of HO on functional activities of the spinal cord injury patient should be studied which can be treatment oriented.
2. Large sample size to show any statistical significance of the biomarkers in identifying in the early stage or even before the onset of HO formation.
3. Identifying the more HO specific bone morphogenic proteins and relevant new bone forming markers which are economically feasible.

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ANNEXURE

Annexure

1. **Institutional Review Board Acceptance letter**
2. **Patient Information Sheet and Consent Form**
3. **Proforma of the study**
4. **Study patient data sheet**
5. **SOP of CPK**
6. **SOP of ALP**
7. **SOP of Osteocalcin**

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

Dr.B.J.Prashantham, M.A.,M.A.,Dr.Min(Clinical)
Director, Christian Counseling Centre
Editor, Indian Journal of Psychological Counseling
Chairperson, Ethics Committee, IRB

Dr. Alfred Job Daniel, MS Ortho
Chairperson, Research Committee &
Principal

Dr. Nihal Thomas
MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

November 13, 2012

Dr. Vijay Kumar Manda,
P.G. Registrar,
Department of Physical medicine and Rehabilitation
Christian Medical College
Vellore 632 004

Sub: **FLUID Research grant project NEW PROPOSAL:**
Role of biomarkers in early detection of heterotopic ossification following Spinal cord injury

Dr. Vijay Kumar Manda, Postgraduate Registrar, Physical medicine and Rehabilitation, Dr. Jacob George, Physical medicine and Rehabilitation, Dr. Joe Fleming, Clinical Biochemistry, Dr. Regi Oommen, Nuclear Medicine

Ref: IRB Min. No 8066 dated 6.11.2012

Dear Dr. Vijay Kumar Manda,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Role of biomarkers in early detection of heterotopic ossification following Spinal cord injury" on November 6, 2012. I am quoting below the minutes of the meeting

The Committee Members raised the following queries

1. Mention the source of your extra funding and what the expenditure is being used for.
2. Investigators contact number and thumb impression slots have to be included.

TEL : 0416 - 2284294, 2284202 FAX : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

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Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. Vijay Kumar Manda and Dr. Jacob George were present during the presentation of the proposal and satisfactorily responded to the queries raised by the Members. After discussion, it was resolved to **ACCEPT the proposal AFTER receiving the suggested modifications and answers to the queries.**

Note:
1. Kindly highlight the modifications in the revised proposal.
2. Keep a covering letter and point out the answer to the queries.
3. Reply to the queries should be submitted with in 3 months duration from the time of the thesis/ protocol presentation, if not the thesis/protocol have to be resubmitted to the IRB.

Email the details to research@cmcvellore.ac.in and send a hard copy through internal dispatch to Dr. Nihal Thomas, Addl. Vice-Principal (Research), Principal's Office, CMC.

Yours sincerely,

Secretary
Institutional Review Board
(Ethics Committee)
Christian Medical College
Vellore - 632 002, Tamil Nadu, India

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

CC: Dr. Jacob George, Physical medicine and Rehabilitation.

TEL : 0416 - 2284294, 2284202 FAX : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Christian Medical College, Vellore
Tamil Nadu, India

This informed consent form is for the adult patients who sustained traumatic spinal cord injury (ages of 18 – 54yr) we have selected to participate in the research to see the effectiveness of various biomarkers to diagnose early the condition of new bone formation (heterotopic ossification).

Name of Principle Investigator	:	Dr. Vijay Kumar Manda **(Ph no. 09442681677, 0416-2282158)**
Name of Sponsors	:	IRB fluid grant and PM&R special fund
Name of Project	:	Role of biomarkers in early detection of heterotopic ossification following spinal cord injury.
Expected duration of the subject's participation	:	From 3rd week to 16th week after traumatic spinal cord injury.

Initial baseline blood investigations like serum creatine phosphokinase, osteocalcin, alkaline phosphatase and baseline X-ray of pelvis and same biomarkers at 7th, 11th and 16th week after injury along with bone scintigraphy at 16th week after injury to be taken for the confirmation of presence of disease.

The subject will not be benefited from the study. However the study results will contribute to the upcoming research on the early prediction of heterotopic ossification, which can potentially affect the rehabilitation and thus arresting the further progression of the condition by appropriate intervention.

Confidentiality:
This study is strictly confidential; the information will not be seen by others. No one will have access to the forms except the project team. All the information about you that will be collected from the research will be put away and no-one but the researchers will be able to see it. Any information about you will have a number on it instead of your name.

You can contact the principle investigator for any queries, rights of subjects and in the event of any injury[1].

Subject's responsibilities on participation: To attend the PM&R OPD as per the requirement especially 7th, 11th and 16th week following the spinal cord injury for the collection of blood samples and bone scan at the end of the study(16th week). Your contact details (mobile/home landline number/e-mail address) will be taken to remind you about your next visit. The subjects are requested to arrange the transport expenses on their own.

The participation in this project is voluntary and the subject can withdraw from the study at any time. The refusal to participate will not involve any penalty or loss of benefits to which the Subject is otherwise entitled[2].

The principle investigator has the right to decide on the termination of the subject's participation depending upon foreseeable circumstances without the subject's consent. Approximate number of subjects enrolled in the study is 20.

Informed Consent form

Study Title: Role of biomarkers in early detection of heterotopic ossification following spinal cord injury

Study Number:

Subject's Initials: _____ Subject's Name: _____

Date of Birth / Age:_____

Please initial box

(Subject)

(i) I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions. [ ]

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

(iii) I understand that the Sponsor of the project, others working on the Sponsor's behalf, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). [ ]

(v) I agree to take part in the above study. [ ]

Signature (or Thumb impression) of the Subject /
Legally Acceptable Representative: _____

Date: _____/_____/_____

Signatory's Name: _____

Signature/thumb of the Investigator: _____

Study Investigator's Name: **Dr. Vijay Kumar Manda (Ph no. 09442681677, 0416-2282158)**
Signature of the Witness (or Thumb impression): _____ Date:_____/_____/_____

Name of the Witness: _____

SOP's for the automated chemistry analyser
CREATINE KINASE (Creatine (phospho)kinase, CK or CPK) EC 2.7.3.2.

Test introduced:	March 1985
Review Date:	Annual
Analyser:	Automated chemistry analysers
Frequency:	available 24 hrs continuously
Analysis time:	urgent results available within 90 mins

Summary and explanation of the test

UV, Kinetic, N-acetyl cysteine activated (NAC) for serum.
This is an optimised standard method conforming to the recommendations of the Deutsche Gesellschalt for Klinische Chemie. NAC presents inactivation of CK. The activity of CK is proportional to the rate of increase of absorbance due to NADPH production.

Clinical Indications for the test

CK is an isoenzyme consisting of 2 protein subunits M and B. The predominant isoenzyme in brain is CK-BB and in muscle CK-MM. CK converts energy stored in creatinine phosphate into ATP for use in situ. CK raises 3-6 hrs after a myocardial infarct and returns to normal in 2-3 days unless a second infarct occurs. It is also increased after heavy exercise and in musclar dystrophies and trauma.

Specimen type, collection and storage

Serum or heparinised plasma may be used. EDTA plasma may cause unpredictable rates.
Activity decrease in serum after 7 days at 4^0C: 2%
24 hrs at 25^0C: 2%
Visible Haemolysis interferes in the test.

Principle of the method

The following is the series of reactions involved:

$$\text{Creatine Phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{Creatine} + \text{ATP}$$

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G-6-P-DH}} \text{6-PG} + \text{NADPH} + \text{H}^+$$

The increase in absorbance at 340 nm, resulting from the formation of NADPH is proportional to the activity of Creatine Kinase in the sample.

HK = hexokinase, G-6-P-DH = Glucose-6-phosphate dehydrogenase

Source of the Method Protocol
Manufacturers kit insert (Roche Diagnostics, GmbH)
DIASYS Diagnostic systems Holzheim GmbH,
Randox , Crumlin, UK

Rec. GSCC (DGKC) J. Clin Chem Clin Biochem 1977; 15: 255.
Szasz G et al. Clin Chem. 1976; 22:650.
Stein W. Med. Welt 1985; 36: 572

Hazardous Reagents

Sodium azide: avoid contact with skin and mucous membranes. Flush affected areas with copious amounts of water. Seek medical attention for eyes or if ingested. Sodium azide may react with lead or copper plumbing to form potentially explosive metal azides. When disposing of, flush away with large quantities of water to prevent azide build up. Clean exposed metal surfaces with 10% sodium hydroxide solution.

Reagents
ROCHE
1. reagent tablets (CK 1a) containing coenzyme, substrate (keep bottle tightly closed)
2. Imidazole buffer (CK 1)

Preparation of reagents **ROCHE**
Dissolve one reagent tablet in 15 ml of buffer as per the manufacturers instructions

Reagent 1: (R1)

Contents	**Concentration in the working solution**
Imidazole buffer	0.1 mol/L, pH 6.7
Glucose	20.0 mmol/L
Mg2+	10.0 mmol/L
EDTA	2.0 mmol/L
HK	$\geq$ 2.5 U/ml
G6P-DH	$\geq$ 1.5 U/ml
ADP	2.0 mmol/L
AMP	5.0 mmol/L
Diadenosine pentaphosphate	10.0 mmol/L
NADP	2.0 mmol/L
Creatine phosphate	30.0 mmol/L
N-acetyl cysteine	20.0 mmol/L

Reagent storage and stability
Working reagent (R1) is stable for seven days at 2-8^{0}C.
12 hours at 15- 25^{0}C.

RANDOX
Reagent 1: (R1a) Buffer/glucose

Contents	**Concentration in the working solution**
Imidazole buffer	0.1 mol/L, pH 6.7
Glucose	20.0 mmol/L
Magnesium Acetate	10.0 mmol/L
EDTA sodium	2.0 mmol/L

Stabilizers and preservatives, sodium azide
Contents ready to use. Stable up to expiry date at 2-8 ^{0}C

Reagent 1: (R1b) Enzymes/Coenzymes/Substrate

Contents	**Concentration in the working solution**
ADP	2.0 mmol/L
AMP	5.0 mmol/
Diadenosine pentaphosphate	10.0 μmol/L
NADP	2.0 mmol/L
HK	≥ 2.5 U/ml
G-6-P-DH	≥ 1.5 U/ml
N-acetyl cysteine	20.0 mmol/L
Creatine phosphate	30.0 mmol/L

Reconstitute one vial of R1b with the appropriate volume of R1a. that is as instructed on the bottle 2.5 ml for a 2.5 ml bottle, 3.0 ml for a 3.0 ml bottle etc.

Reagent storage and stability
Working reagent (R1) is stable for 3 weeks at 2-8^{0}C.
3 days at 15- 25^{0}C

Standards
Boehringer Mannheim (BM), GmbH, calibrator for automated systems (cfas), calibrator serum and solvent. Made from the blood of donors individually tested and free from HbsAg, HCV1 and antibody to HIV 1/2, and subjected to additional heat treatment. Handle according to laboratory safety guidelines, as if potentially infectious.
Calibration performed when quality controls exceed limits (see quality assurance file)

Other required reagents

Sodium chloride (0.9%)
for preparation instructions, refer to the common reagent preparation folder

QC Interval

Two different levels of commercial QC (from Boehringer Mannheim GmbH Precinorm U, BioRad Laboratories Chemistry control and Randox, UK, human control serum level II or similar) are run twice daily, in the morning and in the afternoon

Quality control composition

BM Precinorm U. Universal control serum. Made from the blood of donors individually tested and free from HbsAg, HCV1 antibody to HIV, and subjected to additional heat treatment. Other commercial QC material used is produced in a similar fashion. Handle these QC‘s as if potentially infectious.

External Quality Assessment schemes

For details see quality assurance file

Equipment/Instrumentation
Method

Parameters Roche Modular P800	**RANDOX reagents**
Temperature	37°C
TEST Test number 013	[CK] [323]
ASSAY CODE	[RATE A] [10]—[22]—[34]
SAMPLE VOLUME (µl) (N/DEC/INC)	[3.6—[DF]—[DF]
R1 VOLUME (µl)	[180]—[0]—[0]
R2 VOLUME (µl)	[0]—[0]—[0]
WAVELENGTH (nm)	[376—[340]
CALIB. METHOD	[LINEAR] [2]—[2]—[0]
STD. (1) CONC.-POS.	[0]—water [400]
STD. (2) CONC.-POS.	[ ] calibrator [401] Rack position[S0001-2)
UNIT	[U/L]
SD LIMIT	[0.1]
DUPLICATE LIMIT	[10]% [500] Abs
SENSITIVITY RANGE	[DF]—[DF] DEFAULT (DF)
ABS. LIMIT (**INC)**	[9000]
PROZONE LIMIT	[32000]—[UPPER]
TECHNICAL LIMIT	[0]—[2000]
INSTRUMENT FACTOR	[1.0]

Parameters Olympus ROCHE

Parameter Specific

Test No.	**1**	**Test Name**	**CK**	Sample Type	**serum**		
Sample Vol		**5.0**	Dil Vol		Min OD	Max OD	
Reag 1 Vol		**100**	Dil Vol		L- 2.00	H+2.500	
Reag 2 Vol		**25**	Dil Vol		Linearity 15%		
Wave	Main	**340**	Sub	**410**	Fst. L	Fst, H	
Method	**Rate**	**Slope +**					
Point 1	Fst	**21**	Lst	**27**	Corrl factorA	1.000	

Parameters Olympus RANDOX

Parameter Specific

Test No.	**1**	**Test Name**	**CK**	Sample Type	**serum**		
Sample Vol		**3.0**	Dil Vol		Min OD	Max OD	
Reag 1 Vol		**150**	Dil Vol		L- 2.00	H+2.500	
Reag 2 Vol		**0**	Dil Vol		Linearity 15%		
Wave	Main	**340**	Sub	**410**	Fst. L	Fst, H	
Method	**Rate**	**Slope +**					
Point 1	Fst	**4**	Lst	**14**	Corrl factorA	1.000	

Review of the results and quality control

The senior person in charge of the analyser is responsible for ensuring that quality control values for all assays being run are acceptable. Patient results may be released provided that the relevant QC's are within their allowed limits.

If QC's are outside the limits, patient results cannot be reported. Ascertain whether any error codes have occurred throughout the assay (refer to analyser SOP). Rectify any problem according to SOP instructions and repeat analysis. If there is a repeated failure of QC then call one of the senior staff.

Manufacturers assay range: 2 -1940 U/L

Dilution of high specimens

If value exceeds 19400 U/L. dilute 1 in 10 with saline. Dilute 100µl of specimen with 900µl of 0.9% saline and this figure can be multiplied 10. If this value exceeds 2000 U/L further dilute this diluted specimen 1 in 10 and multiply the final figure x 100.

Interference

No significant effect of haemolysis/haemoglobin < 0.29g/dl

Bilirubin up to 23.5 mg/dl or lipaemia/ triglycerides up to 550 mg/dl. Visible haemolysis invalidates the assay.

Reporting of results

Results reported to	nearest whole number
Units	U/L
Lower limit reporting range	<10 U/L
Phoning limits	not applied
Reference Range	45—195 U/L

Specimen storage and disposal

Blood clots (primary specimen) are stored through the day at room temperature, kept for 3 days at 4°C and then discarded.

All biological specimens are disposed of in accordance with the procedures described in the departmental health and safety manual

Clinical Interpretation of results

Pathological increases and decreases

CK should be assayed 6 hours after the onset of chest pain is suspected myocardial infarction. Infarction is confirmed by any two of ECG changes, chest pain, elevated CK (twice upper reference limit) peak value occurs at 12-24 hrs with return to normal in 3 days.

High levels of cardiac muscle origin: Myocardial infarction, myocarditis .

High levels of skeletal muscle origin: Trauma, injection, muscular dystrophy and miscellaneous causes. Low results are not significant.

This SOP was reviewed by…….

and approved by…….

Date……Number of copies in circulation……

This assay is only to be performed by staffs who have received training to operate the automated chemistry analysers and have read this SOP. The list of these staff is held in the front of the automated chemistries laboratory procedures manual.

No amendment is to be made to this procedure unless agreed and approved by the persons named above, and then written into the procedural amendment form at the front of this manual.

SOP's for Automated Chemistry analysers
Alkaline Phosphatase (ALP) EC 3.1.3.1

Date of introduction: March 1995
Review Date: annual
Analyser: Automated Chemistry analysers
Frequency: daily, Monday to Saturday
Analysis time: results available the same working day, OP up to 1930 hrs

Summary and explanation of the test

Colorimetric, kinetic, p-nitrophenyl phosphate with AMP buffer.
This is an optimised standard method confirming to the recommendation of the IFCC. The liberated phosphate group is transferred to water and the reaction rate is enhanced by certain amino alcohol buffers such as AMP (2 – amino – 2 methyl-1-propanol) which act as a phosphate group acceptor.

Clinical Indications for the test

Phosphatases catalyse the splitting of phosphate group from monophosphoric esters, those operating at a alkaline pH optimum are called ALP. ALP in the serum is a mixture of isoenzymes from liver, bone intestine and placenta, occurring particularly in osteoblasts (bone forming cells) and in the liver. It is increased in bone disease associated with increased osteoblastic activity and in any form of biliary obstruction, which induces enzyme synthesis in hepatocytes adjacent to the biliary canaliculi. It is a standard test for bone and liver disorders.

Specimen type, collection and storage

Serum is the preferred specimin but lithium heparin plasma is also suitable for this test.
Stable for 7 days at 4^0C 0% activity decreased
7 days at 20-25^0C 10% activity decreased

Principle of the method
The substrate is self indicating as the 4-nitrophenol is converted to 4-nitro phenoxide in alkali with increased in absorbance at 415 nm being proportional to the ALP activity.

$$\text{p-nitrophenol phosphate} + H_20 \xrightarrow{\text{ALP}} \text{Phosphate} + \text{p –nitrophenol}$$

Source of the Method Protocol
Tietz NW, Rinker D, Shaw LM. IFCC methods for the measurement of catalytic concentration of enzymes. Part 5 IFCC method for alkaline phosphatase. J. Clin Chem Clin Biochem 1983; 21: 731-748.
Erba Manheim, TransAsia Biomedicals Ltd, Solan

Hazardous Reagents

Hydrochloric acid: corrosive, toxic fumes, open concentrated solution only in fume cupboard. If solution comes in contact with skin or mucous membranes, flush immediately with large quantities of water. If solution comes in contact with eyes, immediately flush liberally with water and consult an ophthalmologist
Paranitrophenol : toxic, do not swallow or inhale avoid contact with skin.

Preparation of reagents

All chemical used are "Analar grade".
Reagent1 (R1) : AMP buffer, 0.35 mmol/L, pH 10.4
In a 500 ml glass beaker add the following :

Zinc sulphate heptahydrate	1.150g
D/L Aspartic Acid, Magnesium salt tetrahydrate	2.88g
EDTA (anhydrous acid)	2.34g

To the above chemicals add 15mls of 6 M HCl and 400 ml of deionised water and mix well with a magnetic stirrer (the chemicals will not dissolve completely).
In a 2L plastic beaker add 136 ml of AMP (free base)
And 1 litre of deionized water. Add the above solution and mix. Now the chemicals will dissolve.
Adjust the pH to 10.4 with approx 10ml of 6M Hcl. Finally make the solution up to 2 litres ibn a 2 litre glass flask.

Reagent 2 (R2)
Dissolve 2.374g of p-nitrophenyl phosphate in 200ml of the above reagent1 and mix well.
Reagent storage and stability
R1 stored in a brown bottle at 4^0C, stable for 4weeks.
R2 stable at 4^0C for 4 weeks

Erba Mannheim

AMP buffer	350 mmol/L,
Magnesium ions	2.0 mmol/L
HEDTA	2.0 mmol/L
p-nitrophenyl phosphate	16.0 mmol/L

Reagent preparation
Reagents R1 and R2 are ready to use. Working reagent is prepared by mixing 4 parts of R1 with 1 part of R2 reagent.
Reagent stability
Stable until expiry date when stored at 2 – 8 ^{0}C. Working reagent is stable for 30 days at 2 – 8 ^{0}C

Standards

Boehringer Mannheim (BM), GmbH, calibrator for automated systems (cfas), calibrator serum and solvent made from the blood of donors individually tested and free from HbsAg, HCV1 and antibody to HIV 1/2, and subjected to additional heat treatment. Handle according to laboratory safety guidelines, as if potentially infectious.
Calibration performed when quality controls exceed limits (see quality assurance file).

Other required reagents
Sodium chloride (0.9%)
Hydrochloric acid 6 mol/L
for preparation instructions, refer to the common reagent preparation folder

QC Interval
Two different levels of commercial quality control material (Boehringer Mannheim GmbH Precinorm U, BioRad chemistry control or Randox, UK, human control serum level II or similar), run twice daily, after instrument start up and each working afternoon.

Quality control composition
Commercial QC sera. Made from the blood of donors and individually tested and free from HbsAg, HCV1 antibody to HIV, and subjected to additional heat treatment. Other QC material made in a similar fashion. Handle all QC's as if potentially infectious.

External Quality Assessment schemes

For details see quality assurance file

Equipment/Instrumentation
Automated Chemistry analysers see instrument SOP's for working instructions

Parameters Roche P800 (manual method)

Temperature	37°C
TEST [ALP]	[319]
ASSAY CODE	[RATE - A] [8] — [13]—[19]
SAMPLE VOLUME (µl) SERUM (N/INC/DEC)	[3 —[20] —[9]
R1 VOLUME (µl)	[60]—[60]
R2 VOLUME (µl)	[30]—[30]
R3 VOLUME (µl)	[0—[0]
WAVELENGTH (nm) [2ND/PRI]	[480]—[415]
CALIB. METHOD-LINEAR 2 POINT 2 SPAN	
STD. (1) CONC.-POS.	[0]—water [400]
STD. (2) CONC.-POS.	[ ]—calibrator [401]
UNIT [U/L]	

SD LIMIT	[0.1]
DUPLICATE LIMIT	[10] % [500]Abs
SENSITIVITY LIMIT	[ ] ---- [ ] DEFAULT
ABS. LIMIT (**INC)**	[2500]
PROZONE LIMIT	[32000]—[UPPER]
S1 ABS RANGE	[ ]—[ ] DEFAULT
TECHNICAL LIMIT	[-99999]—[2000]
INSTRUMENT FACTOR	[1.0]

Parameters Olympus

Parameter Specific

Test No.	**6**	Test Name	**ALP**		Sample Type	**serum**		
Sample Vol		**3.0**	Dil Vol		Min OD		Max OD	
Reag 1 Vol		**60**	Dil Vol	**60**	L-	H+		
Reag 2 Vol		**60**	Dil Vol	**60**				
Wave	Main	**410**	Sub	**480**	Fst. L	Fst, H		
Method		**Slope**	**+**					
Point 1	Fst	**13**	Lst	**19**	Corrl factor		A	

Review of the results and quality control

The senior person in charge of the analyser is responsible for ensuring that quality control values for all assays being run are acceptable. Patient results may be released provided that the relevant QC's are within their allowed limits.

If QC's are outside the limits, patient results cannot be reported. Ascertain whether any error codes have occurred throughout the assay (refer to analyser SOP). Rectify any problem according to SOP instructions and repeat analysis. If there is a repeated failure of QC then call one of the senior staff.

Manufacturers assay range

5 – 1500 U/L

Dilution of high specimens

For values > 1500 U/L dilute 100 µl specimen with 900µl of saline and repeat the assay. Multiply the final result by a factor of 10.

Interference

Even very slightly haemolysed specimens are unsuitable. Haemolysis of 0.29g/dL of haemoglobin decreases values by 40%.

Reporting of results

Results reported to	whole number
Units	U/L
Low reporting limit	<5 U/L
Phoning limits	not applicable
Reference Range	40 – 125 U/L adults

Specimen storage and disposal

Blood clots (primary specimen) are stored through the day at room temperature, kept for 3 days at 4°C and then discarded.
All biological specimens are disposed of in accordance with the procedures described in the departmental health and safety manual

Clinical Interpretation of results

Pathological increases and decreases

Reference range for boys up to 13-15 years is up to 362 U/L) and girls 10-12 years up to 332 U/L
In liver disease if ALP is increased < 3 fold there is predominantly hepatocellular damage.
If values of ALP > 3 X increased there is predominantly cholestasis.
High ALP due to heptobiliary disease is caused by cirrhosis, hepatic tumours, other space occupying lesions, cholangitis, extrabiliary obstruction. Liver disease is confirmed by abnormalities of other liver function tests.
In bone disease, increase in ALP is due to Pagets disease, renal osteodystrophy, rickets, osteomalacia, hyperparathyrodisim, bone tumour, osteomyelitis.
Low levels of ALP are uncommon and rarely of any consequence.

This SOP was reviewed by…….

and approved by…….

Date…….

Number of copies in circulation……..

This assay is only to be performed by staff who have received training to operate the chemistry analysers have read this SOP, and the manufacturers kit insert The list of these staff is held in the front of the automated chemistries laboratory procedures manual.
No amendment is to be made to this procedure unless agreed and approved by the persons named above, and then written into the procedural amendment form at the front of this manual

N-MID Osteocalcin

Intended use

Immunoassay for the in vitro quantitative determination of N-MID osteocalcin in human serum and plasma. The determination is used for the control of antiresorptives therapeutic efficiency, e.g. for patients with osteoporosis or hypercalcemia.

*The **e**lectro**c**hemi**l**uminescence **i**mmuno**a**ssay "ECLIA" is intended for use on the Roche Elecsys 1010 and 2010 immunoassay analyzers.*

Summary*[1–10]

Osteocalcin, the most important non-collagen protein in bone matrix, is a bone-specific, calcium-binding protein which is dependent on vitamin K. It contains 49 amino acids and has a molecular weight of approx 5800 D. It contains up to three Ö-carboxyglutamic acid residues (bone-GLA-protein, BGP).

During bone synthesis osteocalcin is produced by the osteoblasts. Its production is dependent upon vitamin K (formation of Ö-carboxyglutamic acid residues) and is stimulated by vitamin D3. After release from the osteoblasts, osteocalcin is not only assimilated into the bone matrix but also secreted into the blood stream. Accordingly, the serum (or plasma) osteocalcin level is related to the rate of bone turnover in various disorders of bone metabolism, e.g. osteoporosis in particular, but also in primary and secondary hyperparathyroidism or Paget's disease. Osteocalcin is therefore termed a bone turnover marker and is used for this purpose. By means of osteocalcin measurements it is possible to monitor therapy with antiresorptive agents (bisphosphonates or hormone replacement therapy, HRT) in, for example, patients with osteoporosis or hypercalcemia.

Both intact osteocalcin (amino acids 1–49) and the large N-Mid fragment (amino acids 1–43) occur in blood. Intact osteocalcin is unstable due to protease cleavage between amino acids 43 and 44 . The N-Mid-fragment resulting from cleavage is considerably more stable.

The Elecsys N-MID Osteocalcin assay uses two monoclonal antibodies specifically directed against epitopes on the N-Mid-fragment and the N-terminal fragment. The test is non-dependent on the unstable C-terminal fragment (amino acids 43-49) of the osteocalcin molecule and thus ensures constant measurement results under routine conditions in the laboratory.

***Tris(2,2'-bipyridyl)ruthenium(II) complex (Ru(bpy){)*

Test principle*

Sandwich principle. Total duration of assay: 18 minutes

- ***1st incubation**: 20 µl of sample, a biotinylated monoclonal N-MID osteo-calcin-specific antibody and a monoclonal N-MID osteocalcin- specific antibody labeled with a ruthenium complex** react to form a sandwich complex.*
- ***2nd incubation**: after the addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.*
- *The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.*
- *Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve pro-vided via the reagent bar code.*

Reagents - contents and concentrations*

Elecsys N-MID Osteocalcin reagent kit, Cat. No. 2149133 - 100 tests

M Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 ml:

Streptavidin-coated microparticles, 0.72 mg/ml, binding capacity: 470 ng biotin/mg microparticles; preservative.

R1 Anti-N-MID Osteocalcin Ab~biotin (gray cap), 1 bottle, 10 ml:

Biotinylated monoclonal anti-N-MID Osteocalcin antibodies (mouse) 1.5 mg/l; phosphate buffer 100 mmol/l, pH 6.0; preservative.

R2 Anti-N-MID Osteocalcin Ab~Ru(bpy){ (black cap), 1 bottle, 10 ml:

Monoclonal anti-N-MID Osteocalcin antibodies (mouse) labeled with ruthenium complex 1.3 mg/l; phosphate buffer 100 mmol/l, pH 6.0; preservative.

Precautions and warnings

For in vitro diagnostic use, exercise the normal precautions required for handling all laboratory reagents.

Reagent handling*

The reagents in the kit have been assembled into a ready-for-use unit that cannot be separated. All information required for correct operation is read in automatically via the reagent bar code.

Storage and stability*

Store at 2–8°C.

Store the Elecsys N-MID Osteocalcin reagent kit ***upright*** *in order to ensure complete availability of the microparticles during the automatic mixing prior to use.*

Stability:

Unopened at 2–8°C after opening up to the stated expiration date twelve weeks at 2–8°C

After opening twelve weeks at 2-8°C

On the Elecsys 2010 eight weeks

On the Elecsys 1010 eight weeks (stored alternately in the refrigerator and on the analyzer – ambient temperature 20-25°C; upto 20 hours opened in total)

Specimen collection and preparation*

Serum collected using standard sampling tubes.

Li-heparin treated plasma and K_3-EDTA treated plasma.

Note: Avoid haemolysis! Erythrocytes contain proteases which degrade osteocalcin. It is recommended that blood be centrifuged immediately. Stability of serum and heparinized plasma: 8 hours at 15–25°C, three days at 2–8°C, 3 months at –20°C. Freeze only once.[11]

Stability of EDTA-plasma: 2 days at 15–25°C, 3 days at 2–8°C, 3 months at –20°C. Freeze only once.[11]

Samples containing precipitates must be centrifuged before performing the assay. Samples and controls stabilized with azide cannot be used.

Elecsys N-MID Osteocalcin testing procedure*

Materials provided

Cat. No. 2149133, Elecsys N-MID Osteocalcin reagent kit for 100 tests contains:

- *M Streptavidin-coated microparticles*
- *R1 Anti-N-MID Osteocalcin Ab~biotin*
- *R2 Anti-N-MID Osteocalcin Ab~Ru(bpy){*

Materials required (but not provided)

- *Cat. No. 1972111, Elecsys N-MID Osteocalcin CalSet, for 10 calibra-tions*
- *Cat. No. 1972227, PreciControl Bone, for 2 x 2 ml each of PreciControl Bone 1, 2 and 3*
- *Cat. No. 1732277, Elecsys Diluent Universal, 2 x 18 ml sample diluent*
- *Elecsys 1010 or 2010 analyzer*
- *Cat. No. 1662988, Elecsys ProCell, 6 x 380 ml system buffer*
- *Cat. No. 1662970, Elecsys CleanCell, 6 x 380 ml measuring cell cleaning solution*
- *Cat. No. 1706829, Elecsys 1010 Assay Cup, 12 x 32 reaction vessels, or*

Cat. No. 1706802, Elecsys 2010 Assay Cup, 60 x 60 reaction vessels

- *Cat. No. 1706799, Elecsys 2010 Assay Tip, 30 x 120 pipette tips*
- *General laboratory equipment*

Only available in the USA:

• Cat. No. 2144093, Elecsys N-MID Osteocalcin CalCheck, for 3 levels.

Assay*

For optimal performance of the assay it is important to follow the dir-ections given for the analyzer used, and to check that the system's inventory of assay materials and other consumables is adequate.

Resuspension of the microparticles before use and the reading in of the test-specific parameters via the reagent bar code take place auto-matically. No manual input is necessary. If in exceptional cases the bar code cannot be read, enter the 15-digit sequence of numbers.

Elecsys 2010: Bring the cooled reagents to approx. 20°C and place on the reagent disk of the analyzer. Avoid the formation of foam. The system ***automatically*** *regulates the temperature of the reagents and the opening/closing of the bottles.*

Elecsys 1010: Bring the cooled reagents to approx. 20–25°C and place on the sample/reagent disk of the analyzer (ambient temperature 20– 25°C). Avoid the formation of foam. Open bottle caps ***manually*** *before use and* ***close manually*** *after use.*

Calibrators*

Elecsys N-MID Osteocalcin was calibrated against in-house reference standards: osteocalcin in analyte-free human serum matrix.[11]

Every N-MID Osteocalcin reagent set has a bar-coded label containing the specific information for calibration of the particular reagent lot. The predefined master curve is adapted to the analyzer by the use of Elecsys

N-MID Osteocalcin CalSet.

Calibration frequency: Calibration must be performed once per reagent lot using fresh reagent (i.e. not more than 24 hours since the reagent pack was registered on the analyzer). Renewed calibration is recommended as follows:
Elecsys 2010:

- *after one month (when using the same reagent lot)*
- *after seven days (when using the same reagent kit on the analyzer)*

Elecsys 1010:

- *with every reagent kit*
- *after seven days (ambient temperature 20–25°C)*
- *after three days (ambient temperature 25–32°C)*

Both analyzers:

- *as required: e.g. quality control findings outside the specified range. Calibration verification: Not necessary. The analyzer's software auto-matically checks the validity of the curve and draws attention to any deviations.*

Quality control*
Elecsys PreciControl Bone 1, 2 and 3 and other suitable controls. Controls for the various concentration ranges should be run as single de-terminations at least once every 24 hours when the test is in use and after every calibration. The control intervals should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined ranges. Each laboratory should establish guidelines for corrective measures to be taken if values fall outside the range.

Calculation*
Elecsys 1010 and 2010 automatically calculate the N-MID osteocalcin concentration of each sample.
The results are given in ng/ml.
Limitations – interference*[11]
The assay is unaffected by icterus (bilirubin < 65 mg/dl), lipemia (Intralipid < 1500 mg/dl) and biotin < 100 ng/ml (criterion: recovery within ± 10% of initial value).
Hemolysis interferes. Erythrocytes contain proteases which degrade osteocalcin. In patients receiving therapy with high biotin doses (> 5 mg/day) no sample should be taken until at least 8 hours after the last biotin admin-istration.[11]
No influence was observed from rheumatoid factor up to 2200 U/ml. There is no high-dose hook effect at N-MID osteocalcin concentrations up to 4200 ng/ml.
In vitro tests were performed on 16 commonly used pharmaceuticals. No interference with the assay was found.
As with all tests containing monoclonal mouse antibodies, erroneous findings may be obtained from samples taken from patients who have been treated with monoclonal mouse antibodies or have received them for diagnostic purposes. Elecsys N-MID Osteocalcin contains additives which minimize these effects. In rare cases, interference due to extremely high titers of antibodies to streptavidin can occur.
For diagnostic purposes, the Elecsys N-MID Osteocalcin findings should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Measuring (reportable) range*[11]*

0.500–300 ng/ml (defined by the lower detection limit and the maximum of the master curve). Values below the detection limit are reported as < 0.500 ng/ml. Values above the measuring range are reported as > 300 ng/ml (or up to 1500 ng/ml for 5-fold diluted samples).

Dilution

Samples having N-MID osteocalcin concentrations above the measuring range can be diluted with Diluent Universal. The recommended dilution is 1:5 (either automatically by the Elecsys 1010/2010 or manually). The concentration of the diluted sample must be > 60 ng/ml. After manual dilution, multiply the result by the dilution factor. After dilution by the analyzers, the Elecsys 1010/2010 software takes the dilution into account when calculating the sample concentration.

Expected values*[11]*

The expected values range is dependent on the assay type. Until completion of clinical studies with N-MID Elecsys Osteocalcin, the following provisional Enzymun-Test N-MID Osteocalcin results given below are valid (values correspond to the 95th percentile in ng/ml). Lar-ger values are expected when using the Elecsys assay:

	Number	*N-MID osteocalcin*
Women, premenopausal	*200*	*31.2*
Women, postmenopausal, no HRT	*211*	*41.3*
Men, ≥ 50 years	*162*	*26.3*

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data of the test*[11]*

Precision

Representative performance data on the Elecsys analyzers are given below. The results obtained in individual laboratories may differ. Reproducibility was determined using Elecsys reagents, pooled human sera and controls in a modified protocol (EP5-T) of the NCCLS (National Committee for Clinical Laboratory Standards): six times daily for ten days (n = 60). The following results were obtained:

		Intra-assay precision		***Total precision***	
Sample	*Mean ng/ml*	*SD ng/ml*	*%CV*	*SD ng/ml*	*%CV*
Human serum 1	*15.5*	*0.61*	*4.0*	*1.01*	*6.5*
Human serum 2	*13.7*	*0.45*	*3.3*	*0.53*	*3.8*
Human serum 3	*68.3*	*0.92*	*1.4*	*1.22*	*1.8*
PreciControl Bone 1	*21.9*	*0.54*	*2.5*	*0.76*	*3.5*
PreciControl Bone 2	*105.7*	*1.32*	*1.2*	*1.85*	*1.7*
PreciControl Bone 3	*205.5*	*2.45*	*1.2*	*3.54*	*1.7*

Analytical sensitivity (lower detection limit)
< 0.50 ng/ml
The detection limit represents the lowest N-MID osteocalcin concen-tration that can be distinguished from zero. It is calculated as the concen-tration lying two standard deviations above that of the lowest standard (master calibrator, standard 1 + 2 SD, intra-assay precision, n = 21).

Analytical specificity
For the monoclonal antibodies used, the following cross-reactivities were determined:
No cross-reactivity detectable for Ä-CrossLaps, parathyroid hormone or bone-specific alkaline phosphatase.

Method comparison
A comparison of Elecsys N-MID Osteocalcin (y) with a commercially available N-MID osteocalcin test (x) using clinical samples gave the following correlations (ng/ml):
Number of samples measured: 185

Passing/Bablok[12-14]	*Linear regression*
y = -2.79 + 1.29x	*y = -6.24 + 1.43x*
r = 0.987	*r = 0.987*
SD (md68) = 1.54	*Sy.x = 3.28*

The sample concentrations were between approx. 10 and 210 ng/ml (x).

DATA SHEET

S.No	Hno	CPK_3	ALP_3	OsCal_3	CPK_7	ALP_7	OsCal_7	CPK_11	ALP_11	OsCal_11	CPK_16	ALP_16	OsCal_16	Outcome
1	421391F	314	109	14	96	70	9	72	87	18	58	77	22	0
2	648268F	990	133	10	95	111	25	93	95	21	160	84	#NULL!	0
3	657829F	418	106	43	207	111	38	116	127	35	90	169	39	0
4	812058C	179	98	18	79	77	13	124	89	21	92	77	22	0
5	720595F	1,708	167	15	190	115	29	212	126	27	130	108	28	1
6	726942F	82	87	9	47	61	21	93	69	16	98	66	13	0
7	114519A	390	212	11	71	79	24	70	85	31	85	94	28	0
8	754617F	1,190	91	8	99	62	14	129	62	25	497	111	15	1
9	759062F	43	345	14	42	184	21	49	138	30	65	124	30	0
10	902344F	149	344	14	325	112	10	25	521	14	37	329	18	1
11	904850F	352	155	18	117	134	28	78	127	27	74	113	32	0
12	860265F	136	119	15	423	99	21	273	78	26	195	72	15	0
13	909435F	74	136	50	90	130	44	200	246	38	195	162	46	1
14	909819F	304	156	26	446	110	22	226	106	37	119	107	44	0
15	910876F	270	159	18	44	141	32	66	88	30	47	80	27	0
16	979765D	228	184	6	40	93	18	52	79	17	36	116	13	0

HO: 1; No HO: 0